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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/53 — A2

(11) International Publication Number: **WO 00/55624**

(43) International Publication Date: 21 September 2000 (21.09.00)

(21) International Application Number: PCT/CA00/00246

(22) International Filing Date: 9 March 2000 (09.03.00)

(30) Priority Data:
60/124,488 15 March 1999 (15.03.99) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ELISA KIT FOR THE DETERMINATION OF METABOLIC PHENOTYPES

(57) Abstract

The invention relates to an enzyme linked immunosorbent assay (ELISA) kit for the rapid determination of metabolic phenotypes including but not limited to CYP 1A2, N-acetyltamferase-1 (NAT-1), CYP 2P6, CYP 2E1 and CYP 3A4, which can be used on a routine basis in a clinical laboratory. The ELISA kit allows physicians to a) individualize therapy of drugs such as theophylline, tamoxifen, and clozapine and b) to predict susceptibility to carcinogen induced diseases such as colon rectal cancers. To reduce the number of patients undergoing clinical testing by selecting for patients with the appropriate phenotype most likely to respond.

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ELISA KIT FOR THE DETERMINATION OF METABOLIC PHENOTYPES

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to an enzyme linked immunosorbent assay (ELISA) kit for the rapid determination of metabolic phenotypes including but not limited to the following enzymes, CYP 1A2, N-acetyltransferase-1 (NAT-1), CYP 2D6, CYP 2E1, and CYP 3A4. The ELISA kit uses may include but not be limited to, use on a routine basis in a clinical laboratory, and allowing a physician to a) individualize therapy for the numerous drugs metabolized by these enzymes, b) to predict susceptibility to carcinogen induced diseases including many cancers, and c) to reduce the number of patients undergoing clinical testing by selecting for patients with the appropriate phenotype most likely to respond.

(b) Description of the Prior Art

For the majority of drugs (or xenobiotics) administered to humans, their fate is to be metabolized in the liver, into a form less toxic and lipophilic with their subsequent excretion in the urine. Their metabolism involves two systems which act consecutively: the cytochrome P450 system which includes at least 20 enzymes catalyzing oxidation reactions and localized in the microsomal fraction, and the conjugation system which involves at least 5 enzymes. An enzyme of one system can act on several drugs and drug metabolites. The rate of metabolism of a drug differs between individuals and between ethnic groups, owing to the existence of enzymatic

polymorphism within each system. Two or three phenotypes can be distinguished: poor metabolizers (PM), extensive metabolizers (EM), and ultra-extensive metabolizers (UEM). Knowledge of the phenotype is useful clinically because:

a) the phenotype is associated with toxicities in chemical plants, diseases and cancers.

b) it allows physicians to prescribe a drug regimen on the individual basis.

c) it provides a rationale in the design of therapeutic drugs.

Currently, the phenotype is determined by measurements of the molar ratio of metabolites of the drug or a probe drug in the urine samples by high pressure liquid chromatography (HPLC) or capillary electrophoresis (CE), hence using methods which are not readily available in a clinical laboratory.

Drugs metabolized by metabolic enzymes of patent

The enzymes NAT1, CYP1A2, CYP2D6, CYP2E and CYP 3A4 are involved in the metabolism of large number of drugs. Table 1 lists the wide array of medications that are metabolized and the enzymes involved. These include drugs used for a variety of diseases, including asthma (theophylline), malaria (dapsone), breast cancer (tamoxifen), cardiovascular disease (procainimide), organ transplant (cyclosporine), common medications such as painkillers (acetaminophen, codeine), general anesthetics (lidocaine), and anxiolitics (valium). The wide array of medications to which screening is applicable with these enzymes, demonstrates the potential and the impact that a rapid phenotype

screening can have on the outcome and safety of a patient's treatment.

Table 1

Drugs metabolized by xenobiotic enzymes phenotyped by CMPD

Enzyme	Drug
NAT1	p-aminobenzoic acid, p-aminosalicylic acid, dapsone
CYP1A2	Caffeine, theophylline, imipramine, propranolol, clozapine, 17β-estradiol (sex hormone) , urorporhyrinogen, lidocaine, propafenone, tamoxifen (antiestrogen)
CYP2D6	**Psychotropic drugs**: amiflamine, amitryptyline, clomipramine, clozapine, desipramine, haloperidol, imipramine, maprotiline, methoxyphenamine, minaprine, nortriptyline, paroxetine, perphenazine, remoxipride, thioridazine, tomoxetine, trifluperidol, zuclopenthixol. **Cardiovascular agents:** bufuralol, debrisoquine, encainide, flecainide, guanoxan, indoramin, metoprolol, mexiletin, n-propylajmaline, propafenone, propranolol, sparteine, timolol, verapamil. **Miscellaneous agents:** chlorpropamide, codeine, dextromethorphan, methamphetamine, perhexilene, phenformin.
CYP2E1	Ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, p-nitrophenol
CYP3A4	Benzodiazepines, cyclosporin, dextromethorphan dihydropyridines, doxorubicin, erythromycin, etoposide, lidocaine, lovastatin, midazolam, paclitaxel, tamoxifen Calcium Channel Blockers: Nifedipine, Diltiazem, Verapamil.

Associations of metabolic enzymes with altered cancer susceptibility

The metabolic enzymes are responsible for the metabolism of many carcinogenic compounds. Therefore, alterations in the activity of these enzymes alter the biological activity of many carcinogens. Table 2 lists the xenobiotics that are metabolized by the enzymes.

Table 2

Enzymes and the carcinogens they metabolize

Enzyme	Carcinogen
NAT1	diaminobenzidine, N-hydroxy-4-aminobiphenyl; heterocyclic aromatic amines (MeIQx and PhIP)
NAT2	4-aminobiphenyl, diaminobenzidine, heterocyclic aromatic amines (MeIQx, PhIP)
CYP1A2	4-aminobiphenyl, heterocyclic amines (MeIQx, PhIP), 4-methylnitrosamino-1-(3-pyridyl-1-butanone) (NNK, tobacco smoke product)
CYP2D6	Is involved in the metabolism of many carcinogens, however as yet is not reported as the major metabolizer for any
CYP2E1	nitrosodimethylamine, nitrosopyrrolidone, benzene, carbon tetrachloride, 3-hydroxypyridine (tobacco smoke product) .
CYP3A4	N'-nitrosonornicotine (NNN), 4-methylnitrosamino- 1 -(3- pyridyl- 1 -butanone) (NNK), 5-Methylchrysene, 4,4'-methylene-bis(2-chloroaniline) (tobacco smoke products)

Metabolic enzyme phenotypes associated with cancers

The factors influencing cancer development are multi-factorial and it is difficult to associate a cancer with only one cause. However, current research has linked different metabolic phenotypes with increased risk of certain cancers.

Table 3 lists the metabolic enzymes phenotyped by these enzymes and the cancers with which an altered phenotype is linked to an increased susceptibility.

Table 3

Xenobiotic metabolizing enzymes associated with carcinogenesis

Enzyme	Genotype	Cancer	Comments
NAT1	NAT*10	Colorectal	OR = 1,9; 95% CI = 1.2-3.2
		Bladder	Metabolize benzidine
CYP1A2	Fast + Fast NAT2	Colorectal	35% cases vs. 16% controls
CYP2D6	Fast + Slow NAT2	Hepatocellular	OR = 2.6; 95% CI =1.6-4.
CYP2E1	c2	Gastric	OR = 23.6-25.7
CYP3A4	No studies have correlated altered phenotype with altered cancer susceptibility		

NAT1

The NAT1 gene was for a long time classified as monomorphic. However, it is now suggested that NAT1, like the other N-acetyltransferase gene (NAT2), is polymorphic. NAT1 has two phenotypes of slow and rapid metabolizers (e.g. NAT1*4 vs. NAT1*10 genotypes respectively. Measurement of the NAT1 activity is of clinical interest for the following reasons.

Polymorphism

NAT1 is polymorphic and two metabolic phenotypes can be distinguished: rapid, and slow metabolizers. NAT1 metabolizes several drugs and dietary constituents including p-aminobenzoic acid, p-aminosalicylic acid, and dapsone.

In addition, NAT1 activates environmental pro-carcinogens especially diaminobenzidine, N-hydroxy-4-aminobiphenyl; heterocyclic aromatic amines (MeIQx and PhIP). In one study it has been shown that individuals who have the NAT1*10 allele, and hence are rapid N-acetylators, are at a greater risk for colorectal cancer (OR = 1,9; 95% CI = 1.2-3.2, while in another study they have an increased risk for bladder cancer (metabolize benzidine.

Inter Ethnic Differences

The activity of NAT1 varies broadly in a given population. Slow, and rapid NAT1 phenotypes have been distinguished. The NAT1*10 genotype that is associated with rapid metabolic phenotype was monitored in three different ethnic populations, Indian, Malaysian and Chinese. The frequency of NAT1*10 allele was 17%, 39% and 30% respectively. While the NAT1*4 genotype associated with slow metabolizers had a frequency in the same populations of 50%, 30% and 35% respectively. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Dapsone

A classical example of the need for phenotyping in drug dosing is the case of Dapsone. Dapsone is used in the treatment of malaria and is being investigated for the treatment of Pneumocystis carinii pneumonia in AIDS patient. Adverse effects include rash, anemia, methemoglobinemia, agranulocytosis, and hepatic dysfunction. Dapsone is cleared from the body via the NAT1 metabolizing system. A study has shown a correlation between slow acetylation and increased adverse reactions to dapsone. (46% vs. 17% for slow and fast acetylators respectively. For, these reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

It is well known that it is possible to individualize therapy for a large number of drugs (theophylline, digoxin, aminoglycosidases, dapsone etc...). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals NAT1 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the NAT1 phenotyping, which could be accomplished on a

routine basis by any technician with a minimum of training and does not involve complex equipments..

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as dapsone.

CYP 1A2

CYP 1A2 constitutes 15% of the total CYP 450 enzymes in the human liver. Measurement of the CYP 1A2 activity is of clinical interest for the following reasons:

Polymorphism

CYP 1A2 may be polymorphic although it remains to be established firmly. Three metabolic phenotypes can be distinguished: rapid, intermediate and slow metabolizers. CYP 1A2 metabolizes several drugs and dietary constituents including acetaminophen, anti pyrine, 17 β-estradiol, caffeine, cloipramine, clozapine, flutamide (antiandrogenic), imipramine, paracetamol, phenacetin, tacrine and theophylline.

In addition, CYP 1A2 activates environmental pro-carcinogens especially heterocyclic amines and aromatic amines. In one study it has been shown that individuals who are fast N-acetylators and have high CYP 1A2 activity are at a greater risk for colorectal cancer (35% of cases vs. 16% of controls, OR=2.79 (P=0.002).

Induction and Inhibition

CYP 1A2 is induced by a number of drugs and environmental factors such as omeprazole, Iansoprasole, polyaromatic hydrocarbons and cigarette smoke. CYP 1A2

is inhibited by oral contraceptives, ketoconazole, α-napthoflavone, fluvoxamine (seronine uptake inhibitor), furafylline.

Inter Ethnic Differences

The activity of CYP 1A2 varies broadly (60 to 70 fold) in a given population. Slow, intermediate and rapid CYP 1A2 phenotypes have been distinguished. The proportion of these three CYP 1A2 phenotypes varied between ethnic groups and countries: % of intermediates: 50, 70, 60, >95, 60, 20 in U.S.A., African-American, China, Japan, Italy and Australia respectively. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Theophylline

A classical example of the need for phenotyping in drug dosing is the case of Theophylline. Theophylline is used in the treatment of asthma. However, theophylline toxicity continues to be a common clinical problem, and involves life-threatening cardiovascular and neurological toxicity. Theophylline is cleared from the body via the CYP 1A2 metabolizing system. Inhibition of CYP 1A2 by quinolone antibiotic agents or serotonine reuptake inhibitors, may result in theophyline toxicity. For, theses reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

It is well known that it is possible to individualize therapy for a large number of drugs

(theophylline, digoxin, aminoglycosidases, etc.). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals CYP 1A2 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the CYP 1A2 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as theophylline, tamoxifen or clozapine.

CYP 2D6

CYP 2D6 constitutes 1-3% of the total CYP 450 enzymes in the human liver. Measurement of the CYP 2D6 activity is of clinical interest for the following reasons:

Polymorphism

CYP 2D6 was the first P450 enzyme to demonstrate polymorphic expression in humans. Three metabolic phenotypes can be distinguished: poor, PM, extensive (EM) and ultraextensive (UEM) phenotypes. CYP 2D6

metabolizes a large variety of drugs and dietary constituents including:

Psychotropic drugs:

amiflamine, amitryptyline, clomipramine, clozapine, desipramine, haloperidol, imipramine, maprotiline, methoxyphenamine, minaprine, nortriptyline, paroxetine, perphenazine, remoxipride, thioridazine, tomoxetine, trifluperidol, zuclopenthixol.

Cardiovascular agents:

bufuralol, debrisoquine, encainide, flecainide, guanoxan, indoramin, metoprolol, mexiletin, n-propylamaline, propafenone, propranolol, sparteine, timolol, verapamil.

Miscellaneous agents:

chlorpropamide, codeine, dextromethorphan, methamphetamine, perhexilene, phenformin.

In addition, CYP 2D6 is involved in the metabolism of many carcinogens, however as yet is not reported as the major metabolizer for any. In one study it has been shown that individuals who are fast CYP 2D6 metabolizers and slow N-acetylators are at a greater risk for hepatocellular cancer (OR = 2.6; 95% CI =1.6-4.

Induction and Inhibition

CYP 2D6 is inhibited in vitro by quinidine and by viral protease inhibitors as well as by appetite suppressant drugs such as D- and L-fenfluramine.

Inter Ethnic Differences

The activity of CYP 2D6 varies broadly in a given population. Poor (PM), extensive (EM) and ultraextensive (UEM) phenotypes of CYP 2D6 have been distinguished. The PCYP 2D6 gene is inherited as an

autosomal recessive trait and separates 90 and 10% of the white European and North American population into extensive (EM) and poor (PM) metabolizer phenotypes respectively. In another study the percentage of PM in different ethnic populations was observed, and white North Americans and Europeans have 5-10% PM's, American blacks, 1.8%, Native Thais, 1.2%, Chinese 1%, Native Malay population, 2.1%, while the PM phenotype appears to be completely absent in the Japanese population.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Dextromethorphan/ Antidepressants

An example of the need for phenotyping in drug dosing is the case of dextromethorphan. Dextromethorphan is a nonopioid antitussive with psychotropic effects. However, Dextromethorphan doses range from 0 to 6 mg/kg based on individual subject tolerance. Dextromethorphan is activated via the CYP 2D6 metabolizing system. Dextromethorphan produced qualitatively and quantitatively different objective and subjective effects in poor vs. extensive metabolizers (mean performance +/- SE, 95+/-0.5% for EMs vs. 86+/-6% for PMs; $p < 0.05$.

Another important drug for CYP 2D6 phenotyping is the tricyclic antidepressants. For both the PM and UEM phenotypes of CYP2D6 are at risk of adverse reactions. PM individuals given standard doses of these drugs will develop toxic plasma concentrations, potentially leading to unpleasant side effects

including dry mouth, hypotension, sedation, tremor, or in some cases life-threatening cardiotoxicity. Conversely, administration of these drugs to UEM individuals may result in therapeutic failure because plasma concentrations of active drugs at standard doses are far too low. For, these reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

It is well known that it is possible to individualize therapy for a large number of drugs (theophylline, digoxin, aminoglycosidases, dextramethorphan etc.). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals CYP 2D6 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the CYP 2D6 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as dextramethorphan, clozapine or verapamil.

CYP 2E1

CYP 2E1 constitutes approximately 5% of the total CYP 450 enzymes in the human liver. Measurement of the CYP 2E1 activity is of clinical interest for the following reasons:

Polymorphism

There is some evidence of genetic polymorphism of CYP 2E1 in the human population, however, the molecular mechanisms remain to be further characterized. Studies have demonstrated the presence of two alleles, designated c1 and c2. Initial studies have shown a possible linkage of c2 allele to higher CYP 2E1 expression.

CYP 2E1 metabolizes several drugs and dietary constituents including ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, p-nitrophenol.

In addition, CYP 2E1 activates environmental pro-carcinogens especially nitrosodimethylamine, nitrosopyrrolidone, benzene, carbon tetrachloride, 3-hydroxypyridine (tobacco smoke product). In one study it has been shown that individuals who have high CYP 2E1 (c2) activity are at a greater risk for gastric cancer (OR = 23.6-25.7.

Induction and Inhibition

CYP 2E1 is induced by a number of drugs and environmental factors such as cigarette smoke as well as by starvation and in uncontrolled diabetes. CYP 2E1 is inhibited by chlormethiazole, trans-1,2-dichloroethylene and by the isoflavonoids genistein and equol.

Inter Ethnic Differences

The proportion of CYP 2E1 phenotypes varied between ethnic groups and countries: The frequency of the rare c2 allele is about 4% in Caucasians and 20% in the Japanese and a study of a separate polymorphism described a rare C allele that has a frequency of about 10% in Caucasian and 25% in Japanese population. In one study it was shown that Japanese males had much lower levels of CYP2E1 activity as compared to Caucasian males. In another study, it was demonstrated that a Nicaraguan population of mixed white (Spanish) and Asian (central American Indians) origins have an intermediate level of CYP 1A2 allele mutations as compared to the parent populations. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

Acetaminophen

An example of the need for phenotyping in drug dosing is the case of acetaminophen. Acetaminophen is a widely used painkiller. However, acetaminophen causes hepatotoxicity at low frequency. The hepatotoxicity is due to its transformation via CYP 2E1, to a reactive metabolite (N-acetyl-p-benzoquinoneimine) which is capable of binding to nucleophiles. For, these reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

It is well known that it is possible to individualize therapy for a large number of drugs (theophylline, digoxin, aminoglycosidases,

acetaminophen etc...). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals CYP 2E1 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the CYP 2E1 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as acetaminophen.

CYP 3A4

The CYP 3A family constitutes approximately 25% of the total CYP 450 enzymes in the human liver. Measurement of the CYP 3A4 activity is of clinical interest for the following reasons:

Polymorphism

A large degree of interindividual variability in the expression of the CYP 3A4 isoenzymes has been shown in the human liver (>20 fold) however, no genetic basis for this polymorphic expression has been defined to date. CYP 3A4 metabolizes several drugs and dietary

constituents including benzodiazepines, erythromycin, dextromethorphan dihydropyridines, cyclosporin, lidocaine, midazolam, nifedipine, terfenadine cyclosporine A.

In addition, CYP 3A4 activates environmental pro-carcinogens especially N'-nitrosonornicotine (NNN), 4-methylnitrosamino- 1 -(3- pyridyl- 1 -butanone) (NNK), 5-Methylchrysene, 4,4'-methylene-bis(2-chloroaniline) (tobacco smoke products).

Induction and Inhibition

CYP 3A4 is induced by a number of drugs such as dexamethasone, phenobarbital, primidone and the antibiotic rifampicin. Conversely CYP 3A4 is inhibited by erythromycin, grapefruit juice, indinavir, ketoconazole, miconazole, quinine, and saquinavir.

Cyclosporine

An example of the need for phenotyping in drug dosing is the case of cyclosporine in the treatment of organ transplant patients. Cyclosporine is an immunosuppressant administered post transplant to protect the new organ from being rejected. Plasma levels of this drug are critical as high levels lead to renal toxicity but low levels can lead to organ rejection. Cyclosporine is metabolized via the CYP 3A4 system. Several studies have indicated the importance of monitoring CYP 3A4 activity in maintaining an effective and safe cyclosporine dose. For, these reasons, the utility of a reliable phenotyping test is obvious.

Individualized Therapy

It is well known that it is possible to individualize therapy for a large number of drugs

(theophylline, digoxin, aminoglycosidases, cyclosporine etc.). However, individualization of therapy has been extremely slow to develop because the methods used for drug phenotyping involves high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE), which are costly, time consuming, and require expertise not readily applicable in a clinical laboratory.

It would be highly desirable to be provided a method for determining an individuals CYP 3A4 phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for the CYP 3A4 phenotyping, which could be accomplished on a routine basis by any technician with a minimum of training and does not involve complex equipments.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy of drugs such as cyclosporine.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide an enzyme linked immunosorbent assay (ELISA) kit for the rapid determination of metabolic enzyme phenotype, which can be used on a routine basis in a clinical laboratory.

Another aim of the present invention is to provide an ELISA kit which allows a physician to:

a) individualize therapy of drugs metabolized by these enzymes

b) to predict susceptibility to carcinogen induced diseases such as various cancers.

Another aim of the present invention is to provide a method for determining an individual's metabolic enzyme phenotype using a non-toxic drug so as to predict his/her response and side effects profile to a wide range of potentially toxic drugs.

The ELISA phenotyping kits will use non-toxic probe drugs for the determination of the individuals spectrum of metabolic enzyme phenotypes. Table 4 lists the probe drugs that are to be used for each of the proposed enzymes.

Table 4

Enzymes and probes drugs

Enzyme	Probe Drug
NAT1	p-aminosalicylic acid
CYP1A2	Caffeine
CYP2D6	Dextromethorphan
CYP2E1	Chlorzoxazone
CYP3A4	Dextromethorphan

These drugs are consumed by the individual to be phenotyped, and the individuals urine collected 4 hours after consumption. The urine will be analyzed via the ELISA technology developed in the present invention. The urine samples will be monitored for the following probe drug derivatives (Figs. 1-7), and the molar ratios calculated to reveal the individual phenotypes.

In Examples I and II, a detailed description of the probe drug derivatives and the ELISA development

for CYP 1A2 are illustrated. The materials and methods, and the overall general process described for the development of the CYP1A2 ELISA kit for metabolic phenotyping can be and will be applied to the development of the metabolic phenotyping ELISA kits for NAT1, CYP2D6, CYP2E1 and CYP3A4.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates p-aminosalicylic acid derivatives for NAT1 phenotyping by ELISA;

Fig. 2 illustrates caffeine derivatives for CYP1A2 phenotyping by ELISA;

Fig. 3 illustrates 1,7dimethylxanthine derivatives for CYP1A2 phenotyping by ELISA;

Fig. 4 illustrates 1,7dimethyluric acid derivatives for CYP1A2 phenotyping by ELISA;

Fig. 5 illustrates dextromethorphan derivatives for CYP2D6 phenotyping by ELISA;

Fig. 6 illustrates chlorzoxazone derivatives for CYP2E1 phenotyping by ELISA;

Fig. 7 illustrates dextromethorphan derivatives for CYP3A4 phenotyping by ELISA;

Fig. 8 illustrates the synthetic routes for the production of caffeine and 1,7-dimethylxanthine derivatives for CYP1A2 phenotyping in accordance with one embodiment of the present invention;

Fig. 9 illustrates the synthetic routes for the production of caffeine and 1,7-dimethyluric acid derivatives for CYP1A2 phenotyping in accordance with one embodiment of the present invention; and

Fig. 10 illustrates a pattern of samples to be pipetted in a Falcon 96-well microtest tissue culture plate.

DETAILED DESCRIPTION OF THE INVENTION

Different probe drugs can be used to determine the CYP 1A2 phenotype (caffeine, theophylline) In accordance with the present invention suitable probe drugs include with out limitation, caffeine, theophylline or acetaminophen.

Of these caffeine is the preferred probe. Caffeine is widely consumed and relatively safe. In previous studies the phenotype has been generally determined from the ratios of 1,7-dimethylxanthine (1,7 DMX) + 1,7-dimethyluric acid (1,7 DMU) and 1,3,7-trimethylxanthine (1,3,7 TMX, caffeine). In these studies, the subjects are given an oral dose of a caffeine containing-substance, and the urinary concentrations of the target metabolites determined by HPLC (Kilbane, A. J. et al. (1990) Clin. Pharmacol. Ther **47:** 470-477; Tang, B.-K. et al. (1991) Clin. Pharmacol. Ther **49:** 648-657) or CE (Meachers et al. (1998) Biomarkers **3:** 205-218).

Inhibition of CYP 1A2 by quinolone antibiotic agents or serotonine reuptake inhibitors, may result in theophyline toxicity. For, theses reasons, the utility of a reliable phenotyping test is obvious.

Enzyme linked immunosorbent assays (ELISA) have been successfully applied in the determination of low amounts of drugs and other antigenic compounds in plasma and urine samples and are simple to carry out. We have previously developed an ELISA for N-acetyltransferase-2 (NAT2) phenotyping using caffeine as a probe drug (Wong, P., Leyland-Jones, B., and Wainer, I.W. (1995) J. Pharm. Biomed. Anal. **13:** 1079-1086). We have subsequently tested and proven the

validity of the ELISA for the NAT2 phenotyping (Leyland-Jones et al. (1999) Amer. Assoc. Cancer Res. **40:** Abstract 356). The ELISA for NAT2 phenotyping is simpler to carry out than the HPLC and CE.

In accordance with the present invention, there are currently being developed antibodies to measure the molar ratio of caffeine and two caffeine metabolites (1,7-dimethylxanthine (1,7 DMX), 1,7-dimethyluric acid (1,7 DMU)) in urine samples of an individual collected after caffeine consumption. This ratio provides a determination of an individual's CYP 1A2 phenotype. Subsequently, there will be an antigen enzyme linked immunosorbent assay (ELISA) for measuring this ratio using these antibodies. The antibodies of the present invention can be polyclonal or monoclonal antibodies raised against caffeine and two different metabolites of caffeine, which allow the measurement of the molar ratio of caffeine and these metabolites.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the CYP 1A2 phenotype of the individual as follows:

$$\frac{\text{1,7-dimethylxanthine (1,7 DMX) + 1,7-dimethyluric acid (1,7 DMU)}}{\text{caffeine}}$$

Molar ratios of 4 and 12 separate slow, intermediate and fast CYP 1A2 metabolizers (Butler et al. (1992) Pharmacogenetics **2:** 116-117).

MATERIALS AND METHODS

Materials

N-acetyl-p-aminophenol (acetaminophen), dioxane, formic acid 98-100 % glass redistilled and isobutyl

chloroformate are purchased from A&C American Chemicals Ltd. (Ville St-Laurent, Que. Canada); horse radish peroxidase is purchased from Boehringer Mannheim (Montreal, Que., Canada); ELISA plates (96-well Easy Wash™ modified flat bottom, high binding; Corning glass wares, Corning, NY, USA) and Falcon 96-well microtest tissue culture plate, no. 3072 (Beckton Dickinson Labware, Franklin, NJ, USA) are purchased from Fisher (Montreal, Quebec, Canada); alkaline phosphatase conjugated to goat anti-rabbit IgGs, Keyhole limpet hemocyanin (KLH) is from Pierce Chemical Co. (Rockford, IL, USA); acetic anhydride, acetonitrile HPLC grade, benzylurea, bovine serum albumin (Cat. No A-3803), N-bromosuccinimide, caffeine metabolites; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride solution (EDAC), ethyl 4-bromobutyrate, ethyl 6-bromohexanoate, methyl cyanoacetate, deuterated chloroform ($CDCl_3$) , deuterated dimethylsulfoxide (d_6), deuterated oxide (D_2O), 1,4-diaminobutane, diethanolamine, dimethylformamide, dimethylsulfate, di-tert-butyl dicarbonate, ethyl chloroformate, Freund's adjuvant (complete and incomplete), glutaraldehyde (50 % v/v), 1-methylxanthine, p-nitrophenolphosphate disodium salt, palladium, 10 wt. % (dry basis) on activated carbon, o-phenylenediamine hydrochloride, polyoxyethylene sorbitan monolaurate (Tween 20), porcine skin gelatin, protein A-Sepharose 4B, Sephadex ™ G25 fine, sodium hydride, sodium methoxide, theophylline, tributylamine, Tween ™ 20, are purchased from Sigma-Aldrich (St-Louis, Missouri, USA); Silica gel particle size 0.040-0.063 mm (230-400 mesh) ASTM Emerck Darmstadt, Germany was purchased from VWR (Montreal, Que., Canada).

Dioxane is dried by refluxing over calcium hydride for 4 hours and distilled before use. Other reagents were ACS grade.

Synthetic procedures

The synthetic routes for the production of caffeine, 1,7-dimethylxanthine, 1,7-dimethyluric acid derivatives are shown in Figs. 8 and 9.

Synthesis of 7-ethoxycarboxypentyl-1,3-dimethylxanthine (II)

Compound II is synthesized by a procedure similar to that of Daly et al. (Daly, J.W., Mueller, C., Shamin, M. (1991) Pharmacology, 42: 309-321). 320 mg of theophylline (I) (1.78 mmole) is dissolved in 7 mL of dry dimethylformamide and 290 mg of potassium carbonate (2.1 mmole) is added to the reaction mixture. 358 µL of ethyl 6-bromohexanoate (2.02 mmole) is slowly added and the suspension is heated at 60°C for 14 hours. The suspension is filtered in order to remove the potassium carbonate. After washing the potassium carbonate with some dimethylformamide, the solvent is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in chloroform and the solution is dried over magnesium sulfate ($MgSO_4$). The solvent is evaporated under reduced pressure with a rotary evaporator. 480 mg of the product (slightly yellow oil 1.49 mmole) is obtained, corresponding to a yield of 83.7%.

Synthesis of 7-carboxypentyl-1,3-dimethylxanthine (III)

Compound III is synthesized as follows. 225 mg of compound II (0.7 mmole) is dissolved in 7 mL of dimethylformamide. 4 mL of a 10% NaOH solution is added and the solution is refluxed for 30 min (100-125 °C).

The solvents are evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in 7 mL of water and the solution is acidified to pH 4 with a 6N HCl solution. Cooling the solution at 4^0 C crystallizes the product as needle-like crystals. The crystals are filtered under vacuum trough a 15-mL sintered glass funnel (10-15 ASTM) and dried. 175 mg of the product is obtained (0.595 mmole), corresponding to a yield of 85%.

Synthesis of 7-ethoxycarboxylpentyl-1-methylxanthine (V)

Compound V is synthesized as follows. 116 mg of 1-methyxanthine (IV) (0.7 mmole) is dissolved in 4 mL of dimethylformamide. 129 mg of potassium carbonate (0.93 mmole) is added and the resulting solution is stirred. 125 µL of ethyl-6-bromohexanoate (0.7 mmole) in 0.4 mL dimethylformamide is slowly added in three portions. The reaction mixture is heated at 50 ^{0}C for 1.5 hours and at 65 ^{0}C for 1 hour. After cooling, the suspension is filtered and the filtrate is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The product is purified by flash chromatography on a silica gel column (40 x 1 cm) using an ethyl acetate-hexane solution (9:1, v/v) as the eluent.

Synthesis of 7-carboxypentyl-1-methylxanthine (VI)

Compound VI is synthesized as follows. 31 mg of compound V (0.1 mmol) is dissolved in 1 mL of dimethyl-formamide and 660 µL of a 10% NaOH is added. The resulting solution is refluxed for 30 min (100-120 ^{0}C). After cooling at room temperature, the solvent is

evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in water and acidified to pH 4 with a 6N HCl solution. Upon cooling, the solution yields white needle-like crystals, which are filtered and dried. 23 mg of the product (0.082 mmole) is obtained, corresponding to a yield of 82%.

Synthesis of 6-amino-1-benzyl uracil (IX)

Compound IX is synthesized according to the procedure similar of that of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854) as follows. 8.64g of sodium methoxide (160 mmol) is dissolved in 71mL methanol. The solution is stirred and 7.55g of benzylurea (50 mmol) and 4.71mL methyl cyanoacetate (53.4 mmol) are added. The suspension is refluxed 5.5 hours at 68-70°C and cooled at room temperature. After filtration, the methanol is evaporated under reduced pressure with a rotary evaporator. The residue is dissolved in warm distilled water, and the product is precipitated by acidification to pH 3-4 with glacial acetic acid. After 2 hours (or overnight) at room temperature, the suspension is filtered under vacuum through a sintered glass funnel. The product is washed with water and dried. The yield is 62-65%.

Synthesis of 6-amino-1-benzyl-5-bromouracil (X)

Compound X is synthesized according to the procedure of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854) as follows. 3.2g of 6-amino-1-benzyluracil (15.8 mmol) is dissolved at 100° C in 60 mL acetic acid and 3 mL

acetic anhydride. 2.85 g of N-bromosuccinimide (16 mmol) is added in small portions over the next 30 minutes. The reaction mixture is stirred for 1 hour and cooled at room temperature. The precipitate is filtered and washed with small amount of cold ethanol and dried. 3.36 g of white crystals are obtained (12 mmol), corresponding to a yield of 76%.

Synthesis of 6-amino-1-benzyl-5-[N-4'-aminobutyl)-amino] uracil (XI)

Compound XI is synthesized as follows. 3g of compound X (10.71 mmol) is dissolved in 30 mL of 50% 1,4-diaminobutane (bp 158-160°; d 0.877) in water (v/v) and the solution is stirred overnight at room temperature. The solution is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The resulting oil is dissolved in a minimal amount of ethyl acetate-methanol solution (4:1; v/v) and is purified by dry flash chromatography on a silica gel packed in a sintered glass funnel (150 mL) with ethyl acetate-methanol solutions as the eluents. At each successive fraction, the solvent polarity was increased, varying from 60% ethylacetate/40% methanol to 45% ethylacetate/55% methanol (v/v). The product is isolated as a light yellow oil. The amount of purified product obtained is 1.69g (6.1 mmol), corresponding to a yield of 57%.

Synthesis of 6-amino-1-benzyl-5-[N-4'-tert-butoxycarbonyl-amino]uracil (XII)

Compound XII is synthesized as follows. 1.63g of compound XI (5.9 mmol) is dissolved in 5.4 mL of 1 N NaOH solution. 270 mg of sodium bicarbonate (3.2 mmol) and 2.7 mL of water are added. 5.4 mL of di-tert-butyl dicarbonate solution in isopropanol (1.88g (8.61 mmol)

is dissolved in 5.4 mL isopropanol) is added slowly to the solution of compound XI. After stirring for 3 hours at room temperature, 13.4 mL of water is added and the unreacted di-tert-butyl dicarbonate is extracted twice with 20mL of petroleum ether. The pH of the reaction mixture is adjusted to 7 by the addition of a 10% citric acid solution and the solution is extracted twice with 40mL ethyl acetate. The organic layer is dried over sodium sulfate (Na_2SO_4) and is concentrated under reduced pressure with a rotary evaporator. The product is precipitated by the addition of some light petroleum ether to the concentrated solution. 0.99 g of an off-white crystalline compound XII (2.62 mmol) is obtained corresponding to a yield of 44%.

Synthesis of 6-amino -1- benzyl-5-[(N-4'tert-butoxy-carbonylaminobutyl-N-ethoxycarbonyl)-amino]-uracil (XIII)

Compound XIII is synthesized as follows. 806 mg of compound XII (2.14 mmol) was suspended in 7.5 mL of water and stirred energetically. 0.5 mL of ethyl chloroformate (5.22 mmol) is added. 3.75 mL of a 1N NaOH solution is added drop wise and the resulting solution is stirred at room temperature for 2.5 hours. The white solid product is filtered, washed thoroughly with water and dried. 741 mg of the product is obtained (1.77 mmol), corresponding to a yield of 82.7%.

Synthesis of 6-amino-1-benzyl-5-[(N-4'tert-butoxycarbon-ylaminobutyl-N-ethoxycarbonyl)-amino]-3-methyluracil(XIV)

Compound XIV is synthesized as follows. 712 mg of compound XIII (1.77 mmol) is suspended is 5.8mL of water. 2.3mL of a 1N NaOH solution are added and the resulting solution is heated at 40^0 C and vigorously stirred. 0.23mL dimethylsulfate (2.43 mmol) is slowly

added and the resulting solution stirred at 40 ^{0} C for 1.5 hours. The precipitate, which formed during the reaction, is filtered, washed with water and dried. The product is purified from the precipitate by flash chromatography on a silica gel column (40 x 1cm) using a solution of 4% methanol in dichloromethane as eluent. The product is recrystallized from ethyl acetate. 498 mg of compound XIV (1.15 mmol) is obtained, corresponding to a yield of 65%.

Synthesis of 6-amino-5-[(N-4'tert-butoxycarbonylamino-butyl-N-ethoxycarbonyl)-amino]-3-methyluracil (XV)

Compound XV is synthesized as follows. 440mg of compound XIV (1.02 mmol) is dissolved in 12 mL methanol and mixed with 252mg ammonium formate (4 mmol). 240mg of palladium-on-charcoal (10%) are added under nitrogen atmosphere. The catalytic hydrogenation is performed at room temperature for 3 hours. The catalyst is removed by filtration and the filtrate is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. 341 mg of the product is obtained (0.99 mmol) corresponding to a yield of 97%.

Synthesis of 7-(4' aminobutyl)-1-methyluric acid (XVI)

Compound XVI is synthesized as follows. 300mg of compound XV (0.875 mmol) is dissolved in 4.5mL dry dimethylformamide and mixed with 144 mg of sodium hydride (6 mmol). The mixture is stirred at room temperature for 20 min and at 110-115 ^{0}C for 30 min. The color changes slowly to a dark yellow. After cooling, 6.5mL of water are added and the solution is acidified to pH 0 with a 6N HCl solution. The solvents are evaporated under reduced pressure with a rotary evaporator and a high vacuum pump, and the crude

product is dissolved in a ethyl acetate-methanol solution (1:4, v/v). The inorganic salt is removed by filtration and the yellow filtrate is purified by flash chromatography on a silica gel column (40 x 1 cm) using a solution of ethyl acetate-methanol (3:7, v/v) as the eluent. The fraction containing the pure product was evaporated under reduced pressure with a rotary evaporator. After titration of the residue with isopropanol, the product is obtained as a pale yellow solid. 98.9 mg of the product is obtained (0.391 mmol) corresponding to a yield of 45%.

NMR Spectroscopy

^{1}H NMR spectra of synthesized were obtained using a 500 mHz spectrophotometer (Varian XL 500 mHz, Varian Analytical Instruments, San Fernando, CA, USA).

Conjugation of haptens to bovine serum albumin and keyhole limpet hemocyanin

Caffeine-BSA, 1,7-Dimethylanthine-BSA conjugates are prepared by procedure similar to that of Rojo et al. (Rojo et al. (1986) J Immunol. **137:** 904-910). Fifteen mg of BSA is dissolved in 6 mL of a caffeine derivative (or 1,7-dimethylxanthine derivative) solution (1.25 µmoles/mL of water) in a 25-mL erlenmeyer flask followed by the addition of 1.43 mL of an EDAC solution (10 mg/mL of water). The solution is stirred overnight at room temperature and dialyzed against 500 mL water at room temperature for 48 h with two changes per day of the water. The conjugates are stored as 0.5 mL-aliquots at -20° C. The 1,7-Dimethyluric acid conjugate is prepared by the method of Peskar et al. (Peskar (1972) Eur. J. Biochem. **26:**

191-195). 7.5 mg of 1,7 dimethyluric acid (0.03 mmole) is placed in a 5 mL round bottom flask and is dissolved with 1 mL of a 0. 1M Na_2PO_4-NaH_2PO_4 buffer, pH 7.0. A volume of 500 µL of a 0.021 M glutaraldehyde solution (42.5 µL 50 % glutaraldehyde (v/v) per 10 mL of water) is added to the stirred solution. After stirring for 2 hours, 100 µL of a 1M lysine in 0. 1M Na_2PO_4-NaH_2PO_4 buffer, pH 7.0 is added. The solution is stirred for one hour and dialyzed against 250 mL of a 150 mM NaCl, 5 mM Na_2PO_4-NaH_2PO_4 buffer, pH 7.0 for 48 hours with 2-3 changes per day of the buffer. Solution of 1,7-dimethyluric acid-BSA conjugate was stored as 0.5 mL aliquots at -20^0 C. Caffeine-KLH and 1,7-dimethylxanthine-KLH conjugates are prepared as follows. 20 mg of lyophilized powder of KLH is dissolved with 2 mL of a 0.9 M NaCl solution and dialyzed against 100 mL for 10 hours with 2 changes of the solution. To 1.1 mL KLH solution (approximately 10 mg/mL) in a 25-mL erlenmeyer flask, is added 0.8 mL of the caffeine derivative or the 1.7-dimethylxanthine derivative (2.5 µmol/mL of a 0.9 M NaCl). 2 mL of an EDAC solution (10 mg/mL of 0.9 M NaCl), and 1.8 mL 0.9 M NaCl solution are successively added to the derivative solution. The solution is stirred overnight (20 hours) at room temperature. The solution is dialyzed against 250 mL of a 0.9 M NaCl solution for 48 hours with 2-3 changes of the solution per day. The caffeine-KLH and 1.7-dimethylxanthine-KLH solutions are stored as 0.5 mL aliquots at -20^0 C. The 1,7-dimethyluric acid-KLH conjugate is prepared according to a method similar to that of Peskar et al. (Peskar

(1972) Eur. J. Biochem. **26:** 191-195). 20 mg of lyophilized powder of KLH is dissolved with 2 mL of a 0.9 M NaCl solution and dialyzed against 100 mL for 10 hours with 2 changes of the solution. 7.3 mg of 1,7 dimethyluric acid (approximately 0.03 mmole) is placed in a 5 mL round bottom flask and is dissolved with 1 mL of a KLH solution A volume of 500 µL of a 0.021 M glutaraldehyde solution (42.5 µL 50 % glutaraldehyde (v/v) per 10 mL of water) is added dropwise to the stirred solution. After stirring for 2 hours, 100 µL of a 1M lysine in 0. 1M Na_2PO_4-NaH_2PO_4 buffer, pH 7.0 is added. The solution is stirred for one hour and dialyzed against 250 mL of a 0.9M NaCl, 5 mM Na_2PO_4-NaH_2PO_4 buffer, pH 7.0 for 48 hours with 2-3 changes per day of the buffer. Solution of 1,7-dimethyluric acid-BSA conjugate was stored as 0.5 mL aliquots at -20^0 C.

Protein Determination by the method of Lowry et al

(Lowry, O.H. et al. (1951) J. Biol. Chem., **193:** 265-275)

Solutions

Solution A: 2g Na_2CO_3 is dissolved in 50 mL water, 10 mL of 10% SDS and 10 mL 1N NaOH, bring to 100 mL volume with water. Freshly prepared.

Solution B: 1% NaK Tartrate

Solution C: 1% $CuSO_4$.5H2O

Solution D: 1N phenol (freshly prepared): 3mL Folin & Ciaocalteu's phenol reagent (2.0 N) and 3 mL water

Solution E: 98 mL Solution A, 1 mL Solution B, 1 mL Solution C. Freshly prepared

BSA: 1 mg/mL. 0.10 g bovine serum albumin (fraction vol.)/100 mL.

Assay

Standard curve	Tube # (13 x 100mm)						
Solution	1	2	3	4	5	6	7
BSA (µl)	0	10	15	20	30	40	50
Water (µl)	200	190	185	180	170	160	150
Solution E (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Vortex and leave 10 min at room temperature.

Solution D (µl)	200	200	200	200	200	200	200

Vortex and leave at room temperature for 1 hour.

Read absorbance at 750 nm using water as the blank.

Unknown

Solution D.F[a] (in triplicate)	Tube # (13 x 100 mm)			
Unknown (µl)	x	x	x	
Water (µl)	y	y	y	x + y = 200 µl
Solution F (mL)	2.0	2.0	2.0	

Vortex and leave 10 min at room temperature.

Solution D (µl)	200	200	200	200	200	200	200

Vortex and leave at room temperature for 1 hour.

Read absorbance at 750 nm using water as the blank.

Calculate the protein concentration using the standard curve and taking in to account the D.F. (dilution factor) of the unknown

a: D.F. (dilution factor): has to be such that the absorbance of the unknown at 750 nm is with in the range of absorbance of the standards.

Methods to determine the amounts of moles of caffeine , 1,7-DMX or 1,7-DMU incorporated per mg of KLH

This method gives an approximate estimate. It is useful because it allows the determination of whether the coupling proceeded as expected.

A) Solutions

- 10 % sodium dodecyl sulfate (SDS) solution
- 1 % SDS solution
- 0.5 or 1 mg/mL of caffeine-KLH (or 1,7-DMX-KLH or 1,7-DMU-KLH) in a 1 % SDS solution (1 mL).
- 0.5 or 1 mg/mL KLH in a 1 % SDS solution.

B) Procedure

- Measure the absorbance of the caffeine-KLH conjugate (or 1,7-DMX-KLH or 1,7-DMU-KLH) at the wavelength of absorption maximum of caffeine (or 1,7-DMX or 1,7-DMU) with 1 % SDS solution as the blank
- Measure the absorbance of the KLH solution at the wavelength of absorption maximum of caffeine (or 1,7-DMX or 1,7-DMU).
- Calculate the amount of mole of caffeine (or 1,7-DMX or 1,7-DMU) incorporated per mg of KLH with the following formula:

$$y = \frac{A_{\Delta max}(\text{caffeine} - \text{KLH}) - A_{\Delta max}(\text{KLH})}{\varepsilon_{\Delta max}(\text{caffeine}) X [\text{KLH}]}$$

where:

y is the amount of mole of caffeine/mg of KLH;

$\varepsilon_{\lambda max}$ (caffeine) is the molar extinction coefficient of caffeine at the wavelength of absorption maximum.

Coupling of haptens to horse radish peroxidase

The caffeine and 1,7-dimethylxanthine derivatives and the 1,7-dimethyluric acid derivative (after succinylation with succinic anhydride) were conjugated to horse radish peroxidase (HRP) by the following procedure. Place 0.12 mmol of the derivative in a 5 mL round bottom flask. Pipet 500 µL of dioxane

freshly dried over calcium chloride. Stir the suspension and cool at 10° C in a water bath using crushed ice. Pipet 31 µL isobutylchloroformate (0.24 mmol) (recently opened or purchased) and 114 µL tributylamine (0.47 mmol). Stir for 30 min at 10° C. While stirring, dissolve 13 mg of horse radish peroxidase (HRP) in 2 mL of water and cool the solution at 4° C on crushed ice. After the 30 min. of stirring, pipet 100 µL of a 1N NaOH solution (freshly prepared) at 4° C to the HRP solution and pour the alkaline HRP solution at once in the 5 mL flask. Stir the suspension 4 hours at 10-12° C. Separate the free derivative from the HRP conjugate by filtration on a Sephadex G-25™ fine column (1.6 x 30 cm) equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.0. Collect fractions of 1.0-1.2 mL manually or with a fraction collector. During elution two bands may be observed: the HRP conjugate and a light yellow band behind the HRP conjugate. The HRP conjugate band eluted between fractions 11-16. Pool fractions containing the HRP conjugate in a 15 mL tissue culture with a screw cap. Determine the HRP conjugate concentration at 403 nm after diluting an aliquot (usually 50 µL + 650 µL of buffer).

$$[\text{HRP conjugate}]\ (\text{mg/mL}) = A_{403} \times 0.4 \times \text{D.F.}$$

Record the ultraviolet spectrum (UV) absorption spectrum between 320 and 220 nm. The presence of additional absorption peaks at 280 nm, 280 nm and 290 nm for caffeine-HRP, 1,7-DMX-HRP and 1,7-DMU-HRP

conjugates, respectively, are indicators that the coupling proceeded as expected. After the above measurements, 5 µL of a 4% thiomersal solution is added per mL of caffeine-HRP, 1,7-DMX-HRP or 1,7-DMU-HRP conjugate solution. The conjugates are stored at 4°C.

Antibody Production

Six mature females New Zealand white rabbits (Charles River Canada, St-Constant, Que., Canada) were used for antibody production. The protocol employed in this study was approved by the McGill University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. An isotonic saline solution (0.6 mL) containing 240 µg of KLH conjugated antigen was emulsified with 0.6 mL of a complete Freund's adjuvant. 0.5 mL of the emulsion (100 µg of antigen) was injected per rabbit intramuscularly or subcutaneously. Rabbits were subsequently boosted at intervals of three weeks with 50 µg of antigen emulsified in incomplete Freund's adjuvant. Blood was collected without anticoagulant in a vacutainer tube by venipuncture of the ear 10-14 days after boosting and kept at 4°C. After clotting, centrifugation at 4°C, sodium azide was added to the antisera to a final concentration of 0.001% (1µL of a 1 % sodium azide solution per mL of antisera). Antisera were stored as 0.5 mL aliquots at -20 °C.

Antiserum titers

The wells of a microtiter plate were coated with 10 µg mL^{-1} of bovine serum albumin-caffeine (or 1,7-dimethyl xanthine, 1,7-dimethyluric acid) conjugate in 100 mM sodium carbonate buffer, pH 9.6) overnight at

4^0 C (150 µL/well). They were then washed three times with TPBS (phosphate buffer saline containing 0.05 % Tween 20) using a Nunc Immuno Wash 12 autoclavable. Unoccupied sites were blocked by an incubation with 150 µL/well of TPBS containing 0.05 % porcine gelatin for 2 h at room temperature. The wells were washed three times with TPBS and 150 µL of antiserum diluted in TPBS was added. After 2 h at room temperature, the wells were washed three times with TPBS, and 100 µL of goat anti-rabbit IgGs-alkaline phosphatase conjugate diluted in PBS containing 1% BSA was added. After 1 h at room temperature, the wells were washed three times with TPBS and three times with water. To the wells were added 150 µL of a solution containing $MgCl_2$ (0. 5 mM) and p-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min at room temperature, the absorbency was read at 405 nm with a microplate reader. The antibody titer is defined as the dilution required to change the absorbance by one unit (1 au).

Isolation of IgG antibodies

Rabbit IgG antibodies against KLH conjugates were purified by affinity chromatography on a Protein A-Sepharose 4B column as follows. A 0.9 x 15 cm Pharmacia chromatographic column was packed with Protein A-Sepharose 4B suspension to a volume of 1 mL. The column was washed generously with a 0.01 M Na_2HPO_4-NaH_2PO_4 buffer, pH 8.0 containing 0.15M NaCl (PBS) and then washed with 3-4 mL of a 0.1 M trisodium citrate buffer, pH 3.0. The column was then washed generously with PBS. 1 mL of rabbit antiserum is diluted with 1 mL

PBS, and the resulting solution is slowly applied to the column. The column is washed with 15 mL PBS and eluted with a 0.1 M trisodium citrate buffer, pH 3.0. Three fractions of 2.2 mL were collected in 15-mL graduated tubes containing 0.8 mL of 1 M Tris-HCl buffer, pH 8.5. The purified rabbit IgG antibodies were stored at 4 ^{0} C in the presence at 0.01 % sodium azide.

Competitive antigen ELISA

Buffers and water without additives are filtered trough 0.45 µM millipore filters and kept for one week, except the substrate buffer which was freshly prepared. BSA, antibodies, Tween™ 20 and horse radish peroxidase are added to buffers and water just prior to use. Urine samples are usually collected four hours after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at -20°C as 1-mL aliquots in 1.5-mL microtubes. For the ELISA, the urine samples are diluted with isotonic sodium phosphate buffer, pH 7.5 (310 mosM) to give concentrations of caffeine, 1.7-DMX and 1,7-DMU no higher than 3 x 10^{-6} M in the microtiter plate wells. Wells of the ELISA plate were washed with a Nunc-Immuno wash 12 washer. Sixteen mL of a solution of 6.6 µg ml^{-1} of isolated IgG antibodies is prepared in a 100 mM sodium carbonate buffer, pH 9.6, and 150 µL of this solution is pipetted in each well of a microtiter plate using a eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200µL Flex tips from Brinkmann). After coating the wells with antibodies at 4°C for 20 hours, the wells were washed 3 times with the isotonic sodium phosphate buffer containing 0.05% Tween™ 20 (IPBT) and

properly drained by inverting the plate and absorbing the liquid on piece of paper towel. Thirty mL of a solution of a IPBT solution containing 1 % BSA is prepared and 150 µL of this solution is pipetted in each well using a eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200 µL yellow tips (Sarstedt yellow tips for P200 Gilson Pipetman). After 3 hours at room temperature, the wells were washed 3 times with IPBT solution and drained. Samples of 400 µL for determination of caffeine, 1,7-DMX and 1,7-DMU are prepared in 1.5-mL microtubes using Sarstedt yellow tips and a P200 Gilson Pipetman. e) 200 µL of each sample are pipetted in duplicate in a Falcon 96-well microtest tissue culture plate according to the pattern shown in Figure 10, using Sarstedt yellow tips and a P200 Gilson Pipetman. Using an eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and changing the tips of the eight channel pipet (200µL Flex tips from Brinkmann) at each row, 150 µL of samples are transferred in the corresponding wells of a 96-well ELISA microtiter plate coated with antibodies. After the addition of the samples, the microtiter plates are covered and left standing at room temperature for 2 h. While the plate is left standing the substrate buffer without the hydrogen peroxide and o-phenylenediamine hydrochloride is prepared (25 mM citric acid and 50 mM sodium phosphate dibasic buffer, pH 5.0). The microtiter plate is washed 3 times with the IPBT solution and 3 times with a 0.05% Tween™ solution and drained. 50 µL of hydrogen peroxide and 40 mg of o-phenylenediamine are added to the substrate buffer. One

hundred fifty microliters (150 µL) of the substrate buffer solution is then added to each wells using a eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200µL Flex tips (Brinkmann). The microtiter plate is covered and shaken for 25-30 min at room temperature and the enzymatic reaction is stopped by adding 50 µL/well a 2.5 M HCl solution using an eight channel pipet (Brinkmann Transferpette™-8 50-200 µL) and 200µL Flex tips (Brinkmann). After gently shaking for 3 min., the absorbance is read at 490 nm with a microplate reader.

Standard solutions of Caffeine, 1,7-DMX and 1,7-Dimethyluric acid solutions for ELISA

Prepare a 100 mL stock solution of caffeine, 1,7-DMX and 1,7-DMU acid at concentrations of 6.00×10^{-4} M in the 310 mosM sodium phosphate buffer, pH 7.5 (IPB) in a 100 mL volumetric flask. Stirring the solution to insure complete solubilization.

Store the stock solutions as 1 mL aliquots at -20°C.

On the day of the ELISA, thaw one aliquot and warm up at room temperature.

Prepare the following standard solutions of the above compounds

Standard #	[Compound]	Composition
1	6.00×10^{-4} M	Stock solution
2	2.00×10^{-4} M	200 µL S1 + 400 µL IPB
3	1.12×10^{-4} M	200 µL S1 + 868 µL IPB
4	6.00×10^{-5} M	100 µL S1 + 900 µL IPB
5	3.56×10^{-5} M	60 µL S1 + 951 µL IPB
6	2.00×10^{-5} M	100 µL S2 + 900 µL IPB
7	1.12×10^{-5} M	100 µL S3 + 900 µL IPB
8	6.00×10^{-6} M	100 µL S4 + 900 µL IPB
9	3.56×10^{-6} M	100 µL S5 + 900 µL IPB
10	2.00×10^{-6} M	100 µL S6 + 900 µL IPB
11	1.12×10^{-6} M	100 µL S7 + 900 µL IPB
12	6.00×10^{-7} M	100 µL S8 + 900 µL IPB
13	3.56×10^{-7} M	100 µL S9 + 900 µL IPB
14	2.00×10^{-7} M	100 µL S10 + 900 µL IPB
15	1.12×10^{-7} M	100 µL S11 + 900 µL IPB
16	6.00×10^{-8} M	100 µL S12 + 900 µL IPB
17	3.56×10^{-8} M	100 µL S13 + 900 µL IPB
18	2.00×10^{-8} M	100 µL S14 + 900 µL IPB
19	2.00×10^{-9} M	100 µL S15 + 900 µL IPB
20	2.00×10^{-10} M	100 µL S15 + 900 µL IPB
21	2.00×10^{-11} M	100 µL S15 + 900 µL IPB
22	2.00×10^{-12} M	100 µL S15 + 900 µL IPB
23	2.00×10^{-13} M	100 µL S15 + 900 µL IPB

Antibody Specificity

To ensure accuracy in the ELISA measurement of CYP 1A2 phenotyping, the antibodies must have specificity for their individual caffeine metabolites, with little or no recognition of other derivatives. To ensure their selectivity an ELISA will be performed

with standard solutions of the compounds listed in Table 5. An ideal antibody specificity result is hypothesized with the Table 5 as well.

Table 5

Cross-reactivity of caffeine-Ab, 1,7-DMX-Ab and 1,7-DMU-Ab towards caffeine metabolites and structural analogs

Compound	% Cross-reaction		
	Caffeine-Ab	1,7-DMX-Ab	1,7-DMU-Ab
Caffeine	100	0[a]	0
Xanthine	**0**	**0**	**0**
Hypoxanthine	0	0	0
1-Methyl Xanthine	0	0	0
3-Methyl Xanthine	0	0	0
7-Methyl Xanthine	0	0	0
8-Methyl Xanthine	0	0	0
1.3-Dimethyl Xanthine[b]	0	0	0
1,7-Dimethy Xanthine[c]	0	100	0
3,7-Dimethyl Xanthine[d]	0	0	0
Uric acid	0	0	0
1-Methyluric acid	0	0	0
3-Methyluric acid	0	0	0
7-Methyluric acid	0	0	0
1,3-Dimethyluric acid	0	0	0
1,7-Dimethyluric acid	0	0	100
3,7-Dimethyluric acid	0	0	0
1,3,7-Trimethyluric acid	0	0	0
Guanine	0	0	0
Uracil	0	0	0
AAU[e]	0	0	0
AAMU[f]	0	0	0

AADMU[e]	0	0	0

a, The number 0 indicates either an absence of inhibition or an inhibition no higher than 40% at the highest concentration tested in the ELISA (5×10^{-3} M); concentrations of caffeine, 1,7-Dimethyl Xanthine and 1,7-Dimethyluric acid required for 50% inhibition in the competitive antigen ELISA will be determined; b, 1,3-Dimethyl Xanthine, theophylline; c, 1,7-Dimethyl Xanthine, paraxanthine; d, 3,7-Dimethyl Xanthine, theobromine; e, AAU, 5-acetamido-6-aminouracil; f, AAMU, 5-acetamido-6-amino-3-methyluracil; f, AADMU, 5-acetamido-6-amino-1,3-dimethylxanthine.

RESULTS

Positive creation of antibodies against caffeine, 1,7-DMX, and 1,7-DMU can be seen by antibody titers of 30,000-100,000 as determined by the ELISA, strong precipitation lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin, and low cross-reactivity with other caffeine derivatives. These results constitute positive conditions for the development of a competitive antigen ELISA according to the methods described in the above section entitled Materials and Methods.

In accordance with one embodiment of the present invention, a competitive antigen ELISA will be developed for CYP 1A2 phenotyping using caffeine as the probe drug. Contrary to current methods used for phenotyping, the assay is sensitive, rapid and can be readily carried out on a routine basis by a technician with a minimum of training in a clinical laboratory.

EXAMPLE II

Determination of Caffeine, 1,7-Dimethyl Xanthine (1,7-DMX) and 1,7-Dimethyluric acid (1,7-DMU) in urine samples with the ELISA kit

5

Table 6

Content of the ELISA kit and conditions of storage

Item	Unit	State	Amt.	Storage Conditions
Tween™ 20	1 vial	liquid	250 µL/vial	4°C
H_2O_2	1 vial	liquid	250 µL/vial	4°C
Caffeine-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMX-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMU-HRP	1 vial	liquid	250 µL/vial	4°C
Buffer A	4 vials	Solid	0.8894 g /vial	4°C
Buffer B	6 vials	Solid	1.234 g/vial	4°C
Buffer C	6 vials	Solid	1.1170 g/vial	4°C
Buffer D	6 vials	Solid	0.8082 g/vial	4°C
Plate (Caffeine-Ab)	2	Solid	-	4°C
Plate (1,7-DMX-Ab)	2	Solid	-	4°C
Plate (1,7-DMU-Ab)	2	Solid	-	4°C
Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
Standards (Caffeine)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMX)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMU)	14 vials	Liquid	200 µL	-20°C
1N NaOH	1 bottle	Liquid	15 mL	20°C
1N HCl	1 bottle	Liquid	15 mL	20°C

Dilutions of urine samples for the determinations of [Caffeine], [1,7-DMX] and [1X] by ELISA

10

The dilutions of urine samples required for determinations of caffeine, 1,7-DMX and 1,7-DMU are a function of the sensitivity of the competitive antigen ELISA and of caffeine, 1,7-DMX and 1,7-DMU

concentrations in urine samples. It is suggested to dilute the urine samples by a factor so that AAMU and 1X are about 3 x 10^{-6} M in the well of the microtiter plate.

Table 7

	Microtube #							
Dilution Factor	20x	40x	50x	80x	100x	150x	200x	400x
Solution	1	2	3	4	5	6	7	8
Urine Sample (mL)[a] 10x diluted	500	250	200	125	100	66.7	50	25
Buffer B (mL)	500	750	800	875	900	933.3	950	975

a: Vortex the microtubes containing the urine sample before pipetting.

Store the diluted urine samples at -20°C in a box for microtubes.

Buffer B: dissolve the content of 1 vial B/ 100mL

Determination of [caffeine], [1,7-DMX] and [1,7-DMU] in diluted urine samples by ELISA

Precautions

The substrate is carcinogenic. Wear surgical gloves when handling Buffer E (substrate buffer). Each sample is determined in duplicate. An excellent pipeting technique is required. When this technique is mastered the absorbency values of duplicates should be within less than 5%. Buffers C, D, E are freshly prepared. Buffer E-H_2O_2 is prepared just prior to pipeting in the microtiter plate wells.

Preparation of samples:

Prepare table 8 with a computer and print it. This table shows the contents of each well of a 96 well microtiter plate. Enter the name of the urine sample (or number) at the corresponding well positions in

Table 8. Select the dilution factor (D.F.) of each urine sample and enter at the corresponding position in Table 8. Enter the dilution of each urine sample with buffer B at the corresponding position in Table 8: for example a D.F. of 100 (100μL of 10x diluted urine sample + 900 μL buffer B), enter 100/900. See "Dilutions of urine samples..." procedure described above for the preparation of the different dilutions. Prepare the different dilutions of the urine samples in 1.5 mL microtubes using a styrofoam support for 100 microtubes. Prepare Table 9 with a computer and print it. Using a styrofoam support (100 microtubes), prepare the following 48 microtubes in the order indicated in Table 9.

Table 8

Positions of blanks, control and urine samples in a microtiter plate

Sample	Well #	D.F.	Dil.	Sample	Well #	D.F.	Dil.
Blank	1-2	-		Control	49-50		-
Control	3-4	-		8	51-52		
S1	5-6	-		9	53-54		
S2	7-8	-		10	55-56		
S3	9-10	-		11	57-58		
S4	11-12	-		12	59-60		
S5	13-14	-		13	61-62		
S6	15-16	-		14	63-64		
S7	17-18	-		15	65-66		
S8	19-20	-		16	67-68		
S9	21-22	-		17	69-70		
S10	23-24	-		Control	71-72		-
S11	25-26	-		18	73-74		
S12	27-28	-		19	75-76		
S13	29-30	-		20	77-78		
S14	31-32	-		21	79-80		
S15	33-34	-		22	81-82		
1	35-36			23	83-84		
2	37-38			24	85-86		
3	39-40			25	87-88		
4	41-42			26	89-90		
5	43-44			27	91-92		
6	45-46			28	93-94		
7	47-48			Blank	95-96		-

Table 9

Content of the different microtubes

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	Caffeine/1,7-DMX/1,7-DMU	27	9	Dil. Urine
4	S2	Caffeine/1,7-DMX/1,7-DMU	28	10	Dil. Urine
5	S3	Caffeine/1,7-DMX/1,7-DMU	29	11	Dil. Urine
6	S4	Caffeine/1,7-DMX/1,7-DMU	30	12	Dil. Urine
7	S5	Caffeine/1,7-DMX/1,7-DMU	31	13	Dil. Urine
8	S6	Caffeine/1,7-DMX/1,7-DMU	32	14	Dil. Urine
9	S7	Caffeine/1,7-DMX/1,7-DMU	33	15	Dil. Urine
10	S8	Caffeine/1,7-DMX/1,7-DMU	34	16	Dil. Urine
11	S9	Caffeine/1,7-DMX/1,7-DMU	35	17	Dil. Urine
12	S10	Caffeine/1,7-DMX/1,7-DMU	36	Control	Buffer B
13	S11	Caffeine/1,7-DMX/1,7-DMU	37	18	Dil. Urine
14	S12	Caffeine/1,7-DMX/1,7-DMU	38	19	Dil. Urine
15	S13	Caffeine/1,7-DMX/1,7-DMU	39	20	Dil. Urine
16	S14	Caffeine/1,7-DMX/1,7-DMU	40	21	Dil. Urine
17	S15	Caffeine/1,7-DMX/1,7-DMU	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

Solutions

Buffer C: Dissolve the content of one vial C/50 mL.
Pipet 25 mL of Tween™ 20.

Buffer D: Dissolve the content of one vial D/25 mL.
Pipet 25 mL of Tween™ 20.

0.05% Tween™ 20: Pipet 25 mL of Tween™ 20 in a 100 mL erlenmeyer flask containing 50 mL of water.

2.5N HCl: 41.75 mL of 12N HCl/200 mL. Store in a 250 mL glass bottle

Caffeine-HRP conjugate: Pipet 9 mL of Buffer C in a 15 mL glass test tube. Pipet 90 µL of caffeine-HRP stock solution.

1,7-DMX-HRP conjugate: Pipet 9 mL of Buffer C in a 15 mL glass test tube. Pipet 90 µL of 1,7-DMX-HRP stock solution.

1,7-DMU-HRP conjugate: Pipet 9 mL of the 2% BSA solution in a 15 mL glass test tube. Pipet 90 µL of 1,7-DMU-HRP stock solution.

Buffer E - H_2O_2: Dissolve the contents of 1 vial E-substrate/50 mL water. Pipet 25 µL of a 30% H_2O_2 solution (prepared fresh).

Table 10

Standard solutions of caffeine, 1,7-DMX and 1,7-DMU (diluted with buffer B)

Standard	Caffeine	Standard	1,7-DMX	Standard	1,7-DMU
1	1.12×10^{-4} M	1	1.12×10^{-4} M	1	1.12×10^{-4} M
2	6.00×10^{-5} M	2	6.00×10^{-5} M	2	6.00×10^{-5} M
3	3.56×10^{-5} M	3	3.56×10^{-5} M	3	3.56×10^{-5} M
4	2.00×10^{-5} M	4	2.00×10^{-5} M	4	2.00×10^{-5} M
5	6.00×10^{-6} M	5	6.00×10^{-6} M	5	6.00×10^{-6} M
6	3.56×10^{-6} M	6	3.56×10^{-6} M	6	3.56×10^{-6} M
7	2.00×10^{-6} M	7	2.00×10^{-6} M	7	2.00×10^{-6} M
8	1.12×10^{-6} M	8	1.12×10^{-6} M	8	1.12×10^{-6} M
9	6.00×10^{-7} M	9	6.00×10^{-7} M	9	6.00×10^{-7} M
10	3.56×10^{-7} M	10	3.56×10^{-7} M	10	3.56×10^{-7} M
11	2.00×10^{-7} M	11	2.00×10^{-7} M	11	2.00×10^{-7} M
12	1.12×10^{-7} M	12	1.12×10^{-7} M	12	1.12×10^{-7} M
13	6.00×10^{-8} M	13	6.00×10^{-8} M	13	6.00×10^{-8} M
14	3.56×10^{-8} M	14	3.56×10^{-8} M	14	3.56×10^{-8} M
15	2.00×10^{-8} M	15	2.00×10^{-8} M	15	2.00×10^{-8} M

Conditions of the ELISA

Pipet 50 µL/well of Caffeine-HRP (1,7-DMX-HRP or 1,7-DMU-HRP) conjugate solution starting from the last row. Pipet 50 µL/well of diluted urine samples in duplicate, standards, blank with a micropipet (0-200 µL), starting from well # 96 (see Table 11). Cover the plate and mix gently by vortexing for several seconds. Leave the plate at room temperature for 3 hours. Wash three times with 100 µL/well buffer C, using a microtiter plate washer. Wash 3 times with 100 µL/well 0.05% Tween™ 20 solution. Pipet 150 µL/well of Buffer E - H_2O_2 (prepared just prior to pipeting in the microtiter plate wells). Shake for 20-30 min. at room

temperature using an orbital shaker. Pipet 50 µL/well of a 2.5N HCl solution. Shake 3 min. with the orbital shaker at room temperature. Read the absorbance of the wells with a microtiter plate reader at 490 nm. Print the sheet of data and properly label.

Calculation of the [caffeine], [1,7-DMX] and [1,7-DMU] in urine samples from the data

Draw table 11 with a computer. Using the data sheet of the microtiter plate reader, enter the average absorbance values of blanks, controls (no free hapten present), standards and samples in Table 11. Draw the calibration curve on a semi-logarithmic plot (absorbance at 490 nm as a function of the standard concentrations) using sigma-plot (or other plot software). Find the [AAMU] (or [1X]) in the microtiter well of the unknowns from the calibration curve and enter the data in Table 12. Multiply the [caffeine] ([1,7-DMX] or [1,7-DMU] of the unknown by the dilution factor and enter the result in the corresponding cell of Table 12.

Table 11

Average absorbance values of samples in the microtiter plate

Sample	Well #	A_{490}	Sample	Well #	A_{490}
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

Table 12

caffeine, 1,7-DMX and 1,7-DMU concentrations in urine samples

Sample	D.F.	[Caffeine]	[caffeine] x D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			

Table 13

Composition of the different buffers

Buffer	pH	Composition	Conc. (mM)	[P] (mM)
A	7.50	0.15629 g/100 mL NaH_2PO_4 1.622 g/100 mL $Na_2HPO_4.7H_2O$ 1.778 g/100 mL (total weight)	11.325 60.099	71.424
B	7.50	0.1210191 g/100 mL NaH_2PO_4 1.11309 g/100 mL $Na_2HPO_4.7H_2O$ 1.2341 g/100 mL (total weight)	8.769 41.23	49.999
C	7.50	1 g/ 100mL BSA 0.1210191 g/100 mL NaH_2PO_4 1.11309 g/100 mL $Na_2HPO_4.7H_2O$ 2.2341 g/100 mL (total weight)	8.769 41.23	49.999
D	7.50	2 g/ 100mL BSA 0.1210191 g/100 mL NaH_2PO_4 1.11309 g/100 mL $Na_2HPO_4.7H_2O$ 3.2341 g/100 mL (total weight)	8.769 41.23	49.999
E	5.00	0.52508 g/ 100mL of citric acid 1.34848 g/100 mL $Na_2HPO_4.7H_2O$ 40 mg/100 mL of o-phenylenedi-amine hydrochloride 1.913567 g/100 mL (total weight)	25 50	-

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention

and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of determining CYP 1A2 phenotype of an individual which comprises measuring, molar ratio of caffeine and first and second different metabolites of caffeine in a biological sample of said individual after drinking a caffeine solution with at least three antibodies, each specific to caffeine or a different metabolite of caffeine, wherein a molar ratio of 4 is indicative of slow intermediate and of 12 is indicative of fast CYP 1A2 metabolizers; and whereby said molar ratio is indicative of a CYP 1A2 phenotype of said individual.

2. The method of claim 1, wherein said first caffeine metabolite is selected from the group consisting of 1,7-dimethylxanthine (1,7 DMX), and those illustrated in Fig. 3; wherein said second caffeine metabolite is selected from the group consisting of 1,7-dimethyluric acid (1,7 DMU), and those illustrated in Fig. 4; and wherein said third metabolite is selected from the group consisting of 1,3,7-trimethylxanthine (caffeine) and those illustrated in Fig. 2.

3. The method of claim 2, wherein said biological sample is urine sample.

4. The method of claim 3, wherein said determined CYP 1A2 phenotype of said individual allows physician to predict susceptibility to carcinogen induced disease and/or to individualize drug treatments.

5. A competitive enzyme linked immunosorbent assay (ELISA) method for determining CYP 1A2 phenotype, which comprises using at least three antibodies each specific to caffeine or a different metabolite of caffeine to measure their molar ratio in biological sample of an individual after drinking a caffeine solution; wherein a molar ratio of 4 is indicative of slow intermediate and of 12 is indicative of fast CYP 1A2 metabolizers; and whereby said molar ratio is indicative of a CYP 1A2 phenotype of said individual.

6. The ELISA method of claim 5, wherein said first caffeine metabolite is selected from the group consisting of 1,7-dimethylxanthine (1,7 DMX), and those illustrated in Fig. 3; wherein said second caffeine metabolite is selected from the group consisting of 1,7-dimethyluric acid (1,7 DMU), and those illustrated in Fig. 4; and wherein said third metabolite is selected from the group consisting of 1,3,7-trimethylxanthine (caffeine) and those illustrated in Fig. 2.

7. The ELISA method of claim 6, wherein said biological sample is urine sample.

8. The ELISA method of claim 7, wherein the determined CYP 1A2 phenotype of said individual allows a physician to predict susceptibility to carcinogen induced diseases and/or to individualize drug treatments.

9. A competitive enzyme linked immunosorbent assay (ELISA) kit for determining CYP 1A2 phenotype, which comprises at least three antibodies each specific to caffeine or a different metabolite of caffeine to measure their molar ratio in biological sample of an individual after drinking a caffeine solution; wherein a molar ratio of 4 is indicative of slow intermediate and of 12 is indicative of fast CYP 1A2 metabolizers; and whereby said molar ratio is indicative of a CYP 1A2 phenotype of said individual.

10. The competitive ELISA kit of claim 9, further comprises:

a) a plate coated with a first antibody specific to caffeine;

b) a second antibody specific to a first metabolite of caffeine;

c) a third antibody specific to a second metabolite of caffeine;

d) a known amount of caffeine-horseradish peroxidase conjugate wherein a standard calibration curve is obtained;

e) a known amount of 1,7-dimethyl xanthine-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

f) a known amount of 1,7-dimethyluric acid-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

11. The method of claim 1 wherein said specific antibodies are polyclonal or monoclonal antibodies.

12. The method of claim 1 wherein said specific antibodies are polyclonal antibodies.

13. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 5 wherein said specific antibodies are polyclonal or monoclonal antibodies.

14. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 5 wherein said specific antibodies are polyclonal antibodies.

15. The competitive ELISA kit of claim 10 wherein said specific antibodies are polyclonal or monoclonal antibodies.

16. The competitive ELISA kit of claim 10 wherein said specific antibodies are polyclonal antibodies.

17. A method of determining NAT1 phenotype of an individual which comprises measuring molar ratio of p-aminosalicylic acid in a biological sample of an individual after consuming p-aminosalicylic acid with at least 2 antibodies each specific to p-aminosalicylic acid or a different metabolite of p-aminosalicylic acid, and whereby said molar ratio is indicative of a NAT1 phenotype of said individual.

18. The method of claim 17, wherein a first p-aminosalicylic acid metabolite is selected from the group consisting of 4-oxomethyl-aminosalicylic acid and those illustrated in Fig. 1; wherein p-aminosalicylic acid is selected and illustrated in Fig. 1.

19. The method of claim 18, wherein said biological sample is urine sample.

20. The method of claim 19, wherein said determined NAT1 phenotype of said individual allows physician to predict susceptibility to carcinogen induced disease and/or individualize drug treatments.

21. A competitive enzyme linked immunosorbent assay (ELISA) method for determining NAT1 phenotype, which comprises using at least 2 antibodies each specific to p-aminosalicylic acid or a metabolite of p-aminosalicylic acid to measure their molar ratio in biological sample of an individual after consuming p-aminosalicylic acid; and whereby said molar ratio is indicative of a NAT1 phenotype of said individual.

22. The ELISA method of claim 21, wherein a first p-aminosalicylic acid metabolite is selected from the group consisting of 4-oxomethyl-aminosalicylic acid and those illustrated in Fig. 1; wherein p-aminosalicylic acid is selected and illustrated in Fig. 1.

23. The ELISA method of claim 22, wherein said biological sample is urine sample.

24. The ELISA method of claim 23, wherein the determined NAT1 phenotype of said individual allows a physician to predict susceptibility to carcinogen induced diseases and/or to individualize drug treatments.

25. A competitive enzyme linked immunosorbent assay (ELISA) kit for determining NAT1 phenotype, which comprises at least 2 antibodies each specific to p-aminosalicylic acid or a metabolite of p-aminosalicylic acid to measure their molar ratio in a biological sample of an individual after consuming p-aminosalicylic acid, and whereby said molar ratio is indicative of a NAT1 phenotype of said individual.

26. The competitive ELISA kit of claim 25, further comprises:

a) a plate coated with a first antibody specific to p-aminosalicylic acid;

b) a second antibody specific to a first metabolite of p-aminosalicylic acid;

c) a known amount of p-aminosalicylic acid-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

d) a known amount of p-aminosalicylic metabolite-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

27. The method of claim 17 wherein said specific antibodies are polyclonal or monoclonal antibodies.

28. The method of claim 17 wherein said specific antibodies are polyclonal antibodies.

29. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 21 wherein said specific antibodies are polyclonal or monoclonal antibodies.

30. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 21 wherein said specific antibodies are polyclonal antibodies.

31. The competitive ELISA kit of claim 26 wherein said specific antibodies are polyclonal or monoclonal antibodies.

32. The competitive ELISA kit of claim 26 wherein said specific antibodies are polyclonal antibodies.

33. A method of determining CYP 2D6 phenotype of an individual which comprises measuring molar ratio of first and second different metabolites of dextromethorphan in a biological sample of said individual after consuming dextromethorphan with at least 2 antibodies each specific to dextromethorphan or a metabolite, wherein a molar ratio >1 is indicative of slow intermediate and <1 is indicative of fast CYP 2D6 metabolizers; and whereby said molar ratio is indicative of a CYP 2D6 phenotype of said individual.

34. The method of claim 33, wherein a first dextromethorphan metabolite is selected from the group consisting of 3 hydroxy-17-methylmorphinan, and those illustrated in Fig. 5; and dextromethorphan is selected and illustrated in Fig. 5.

35. The method of claim 34, wherein said biological sample is urine sample.

36. The method of claim 35, wherein the determined CYP 2D6 phenotype of said individual allows physician to predict susceptibility to carcinogen induced disease and/or to individualize drug treatments.

37. A competitive enzyme linked immunosorbent assay (ELISA) method for determining CYP 2D6 phenotype, which comprises using at least 2 antibodies each specific to dextromethorphan or a metabolite of dextromethorphan to measure their molar ratio in a biological sample of an individual after consuming dextromethorphan, wherein a molar ratio >1 is indicative of slow and a molar ratio <1 is indicative of fast CYP 2D6 metabolizers; whereby said molar ratio is indicative of a CYP 2D6 phenotype of said individual.

38. The ELISA method of claim 37, is selected from the group consisting of 3-hydroxy-17-methylmorphinan, and those illustrated in Fig. 5, a dextromethorphan is selected and illustrated in Fig. 5.

39. The ELISA method of claim 38, wherein said biological sample is urine sample.

40. The ELISA method claim 38, wherein the determined CYP 2D6 phenotype of said individual allows a physician to predict susceptibility to carcinogen induced diseases and/or to individualize drug treatments.

41. A competitive enzyme linked immunosorbent assay (ELISA) kit for determining CYP 2D6 phenotype, which

comprises at least 2 antibodies each specific to a different metabolite of dextromethorphan to measure their molar ratio in a biological sample of an individual after consuming dextromethorphan, wherein a molar ratio >1 is indicative of slow and a molar ratio <1 is indicative of fast CYP 2D6 metabolizers; whereby said molar ratio is indicative of a CYP 2D6 phenotype of said individual.

42. The competitive ELISA kit of claim 41, further comprises:

a) a plate coated with a first antibody specific to dextromethorphan;

b) a second antibody specific to a first metabolite of dextromethorphan;

c) a known amount of dextromethorphan-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

d) a known amount of dextromethorphan metabolite-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

43. The method of claim 33 wherein said specific antibodies are polyclonal or monoclonal antibodies.

44. The method of claim 33 wherein said specific antibodies are polyclonal antibodies.

45. The competitive enzyme linked immunosorbent assay (ELISA) of claim 37 wherein said specific antibodies are polyclonal or monoclonal antibodies.

46. The competitive enzyme linked immunosorbent assay (ELISA) of claim 37 wherein said specific antibodies are polyclonal antibodies.

47. The competitive ELISA kit of claim 42 wherein said specific antibodies are polyclonal or monoclonal antibodies.

48. The competitive ELISA kit of claim 42 wherein said specific antibodies are polyclonal antibodies.

49. A method of determining CYP 2E1 phenotype of an individual which comprises measuring molar ratio of first and second different metabolites of chlorzoxazone in a biological sample of an individual after consuming chlorzoxazone with at least 2 antibodies, each specific to a different metabolite of chlorzoxazone, whereby said molar ratio is indicative of a CYP 2E1 phenotype of said individual.

50. The method of claim 49, wherein a first chlorzoxazone metabolite is selected from the group consisting of 5-chloro-6-hydroxy-benzoxazole, and those illustrated in Fig. 6; chlorzoxazone is selected and illustrated in Fig. 6.

51. The method of claim 50, wherein said biological sample is urine sample.

52. The method of claim 51, wherein the determined CYP 2E1 phenotype of said individual allows physician

to predict susceptibility to carcinogen induced disease and/or to individualize drug treatments.

53. A competitive enzyme linked immunosorbent assay (ELISA) method for determining CYP 2E1 phenotype, which comprises using at least 2 antibodies each specific to a different metabolite of chlorzoxazone to measure their molar ratio in a biological sample of an individual after consuming chlorzoxazone; whereby said molar ratio is indicative of a CYP 2E1 phenotype of said individual.

54. The ELISA method of claim 53, wherein a first chlorzoxazone metabolite is selected from the group consisting of 5-chloro-6-hydroxy-benzoxazole, and those illustrated in Fig. 6, and chlorzoxazone is selected and illustrated in Fig. 6.

55. The ELISA method of claim 53, wherein said biological sample is urine sample.

56. The ELISA method claim 53, wherein the determined CYP 2E1 phenotype of said individual allows a physician to predict susceptibility to carcinogen induced diseases and/or to individualize drug treatments.

57. A competitive enzyme linked immunosorbent assay (ELISA) kit for determining CYP 2E1 phenotype, which comprises at least 2 antibodies each specific to a different metabolite of chlorzoxazone to measure their molar ratio in a biological sample of an individual

after consuming chlorzoxazone; whereby said molar ratio is indicative of a CYP 2E1 phenotype of said individual.

58. The competitive ELISA kit of claim 57, further comprises:

a) a plate coated with a first antibody specific to chloroxazone;

b) a second antibody specific to a first metabolite of chlorzoxazone;

c) a known amount of chlorzoxazone -horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

d) a known amount of chlorzoxazone metabolite-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

59. The method of claim 49 wherein said specific antibodies are polyclonal or monoclonal antibodies.

60. The method of claim 49 wherein said specific antibodies are polyclonal antibodies.

61. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 53 wherein said specific antibodies are polyclonal or monoclonal antibodies.

62. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 53 wherein said specific antibodies are polyclonal antibodies.

63. The competitive ELISA kit of claim 58 wherein said specific antibodies are polyclonal or monoclonal antibodies.

64. The competitive ELISA kit of claim 58 wherein said specific antibodies are polyclonal antibodies.

65. A method of determining CYP 3A4 phenotype of an individual which comprises measuring molar ratio of first and second different metabolites of dextromethorphan in a biological sample of an individual after consuming dextromethorphan with at least 2 antibodies, each specific to dextromethorphan or a metabolite of dextromethorphan, whereby said molar ratio is indicative of a CYP 3A4 phenotype of said individual.

66. The method of claim 65, wherein a first dextromethorphan metabolite is selected from the group consisting of 3-methoxy-morphinan, and those illustrated in Fig. 7; and dextromethorphan is selected and illustrated in Fig. 7.

67. The method of claim 66, wherein said biological sample is urine sample.

68. The method of claim 67, wherein the determined CYP 3A4 phenotype of said individual allows physician to predict susceptibility to carcinogen induced disease and/or to individualize drug treatments.

69. A competitive enzyme linked immunosorbent assay (ELISA) method for determining CYP 3A4 phenotype, which comprises using at least 2 antibodies each specific to a different metabolite of dextromethorphan to measure their molar ratio in a biological sample of an individual after consuming dextromethorphan, whereby said molar ratio is indicative of a CYP 3A4 phenotype of said individual.

70. The ELISA method of claim 69, wherein a first dextromethorphan metabolite is selected from the group consisting of 3-methoxymorphinan, and those illustrated in Fig. 7; and dextromethorphan is selected and illustrated in Fig. 7.

71. The ELISA method of claim 69, wherein said biological sample is urine sample.

72. The ELISA method claim 69, wherein the determined CYP 3A4 phenotype of said individual allows a physician to predict susceptibility to carcinogen induced diseases and/or to individualize drug treatments.

73. A competitive enzyme linked immunosorbent assay (ELISA) kit for determining CYP 3A4 phenotype, which comprises at least 2 antibodies each specific to a different metabolite of dextromethorphan to measure their molar ratio in a biological sample of an individual after consuming dextromethorphan, whereby said molar ratio is indicative of a CYP 3A4 phenotype of said individual.

74. The competitive ELISA kit of claim 73, further comprises:

a) a plate coated with a first antibody specific to dextromethorphan;

b) a second antibody specific to a first metabolite of dextromethorphan;

c) a known amount of dextromethorphan-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and

d) a known amount of dextromethorphan metabolite-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

75. The method of claim 65 wherein said specific antibodies are polyclonal or monoclonal antibodies.

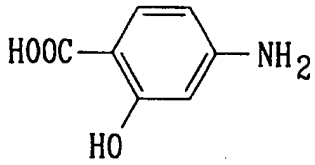
76. The method of claim 65 wherein said specific antibodies are polyclonal antibodies.

77. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 69 wherein said specific antibodies are polyclonal or monoclonal antibodies.

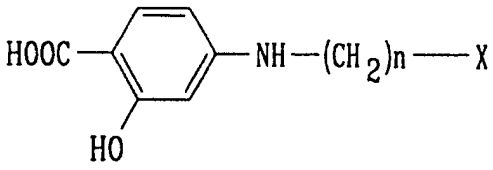
78. The competitive antigen enzyme linked immunosorbent assay (ELISA) of claim 69 wherein said specific antibodies are polyclonal antibodies.

79. The competitive ELISA kit of claim 74 wherein said specific antibodies are polyclonal or monoclonal antibodies.

80. The competitive ELISA kit of claim 74 wherein said specific antibodies are polyclonal antibodies.



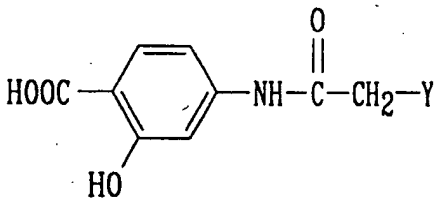
p-aminosalicylic acid



p-aminosalicylic acid derivative I

where X = NH_2
$(CH_2)_nNH_2$
N=C=S
I

where n = 1-5

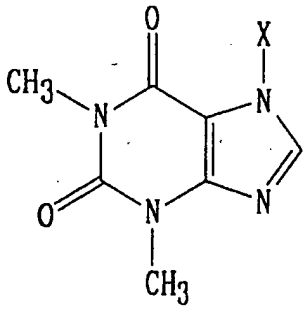


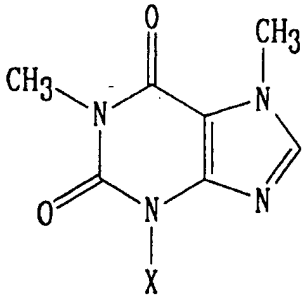
p-aminosalicylic acid derivative II

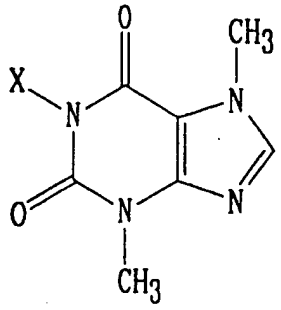
where Y = $(CH_2)_nNH_2$
$(CH_2)_nN=C=S$
$(CH_2)_nOH$
$(CH_2)_nI$

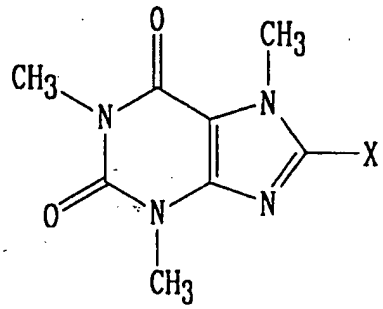
where n = 1-5

Fig. 1









where X = $(CH_2)_nCOOH$ n=1-5

or

$(CH_2)_nNH_2$ n=1-5

or

$(CH_2)_nOH$ n=2-5

or

$(CH_2)_nNHCOCH_2CH_2COOH$ n=1-5

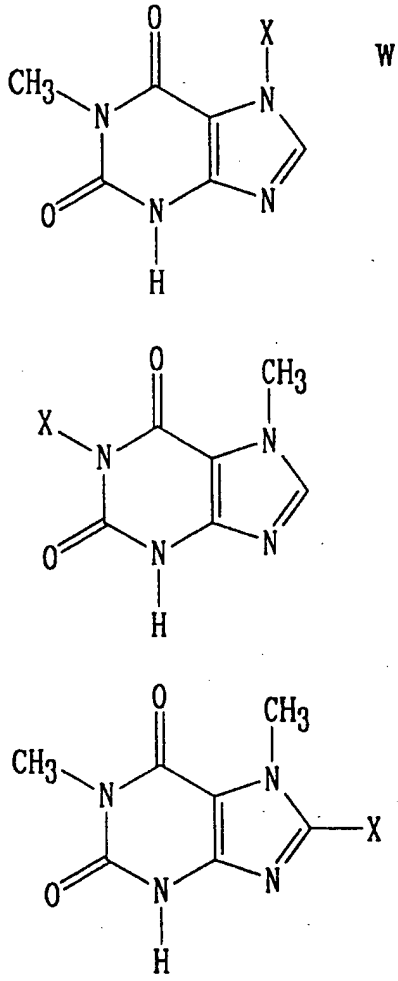
or

$(CH_2)_nOCOCH_2CH_2COOH$ n=2-5

or

$(CH_2)_nN=C=S$ n=1-5

Fig. 2



where X = $(CH_2)_nCOOH$ n=1-5

or

$(CH_2)_nNH_2$ n=1-5

or

$(CH_2)_nOH$ n=2-5

or

$(CH_2)_nNHCOCH_2CH_2COOH$ n=1-5

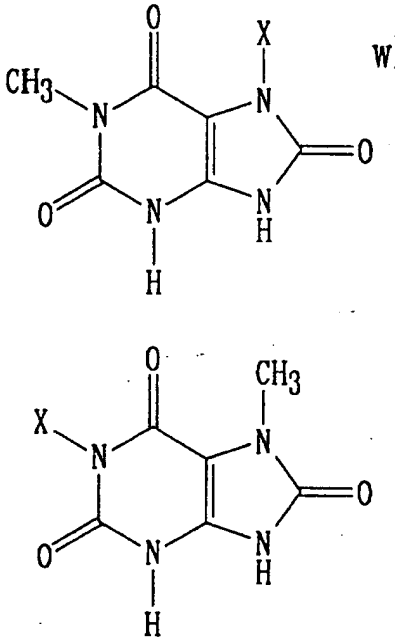
or

$(CH_2)_nOCOCH_2CH_2COOH$ n=2-5

or

$(CH_2)_nN=C=S$ n=1-5

Fig. 3



where X = $(CH_2)_nCOOH$ n=1-5

or

$(CH_2)_nNH_2$ n=1-5

or

$(CH_2)_nOH$ n=2-5

or

$(CH_2)_nNHCOCH_2CH_2COOH$ n=1-5

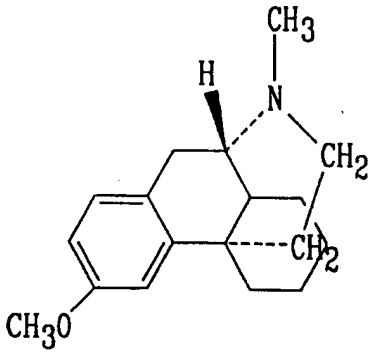
or

$(CH_2)_nOCOCH_2CH_2COOH$ n=2-5

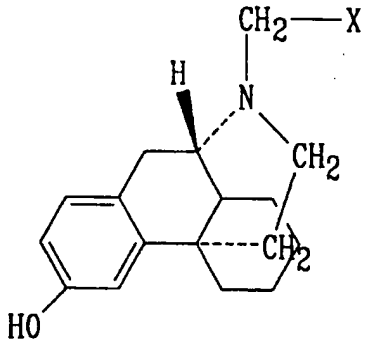
or

$(CH_2)_nN=C=S$ n=1-5

FIG - 4



Dextromethorphan



Dextromethorphan derivative I

where X = $(CH_2)_nCOOH$

$(CH_2)_nNH_2$

$(CH_2)_nNHCOCH_2CH_2COOH$

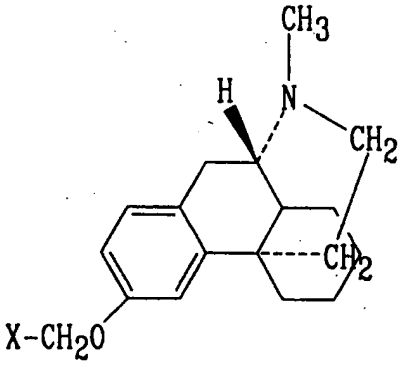
$(CH_2)_nN=C=S$

$(CH_2)_nOH$

$(CH_2)_nOCOCH_2CH_2COOH$

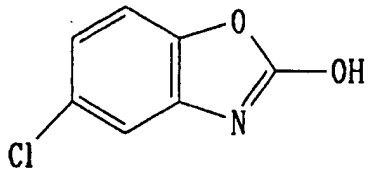
CH_2I

where n = 1-5

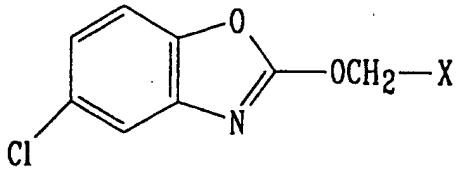


Dextromethorphan derivative II

Fig 5



Chlorzoxazone



Chlorzoxazone derivative I

where X = $(CH_2)_nNH_2$

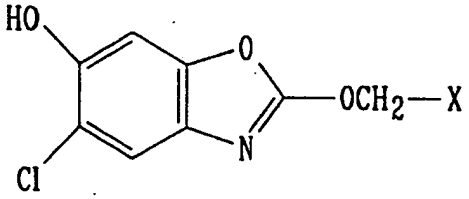
$(CH_2)_nN=C=S$

$(CH_2)_nOH$

$(CH_2)_nOCO-CH_2CH_2COOH$

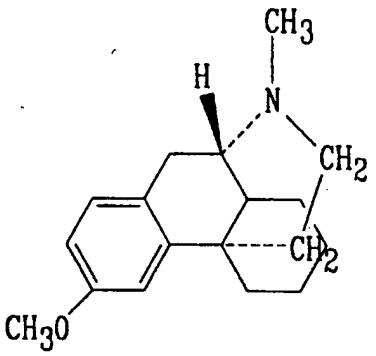
$(CH_2)_nI$

where n = 0-5

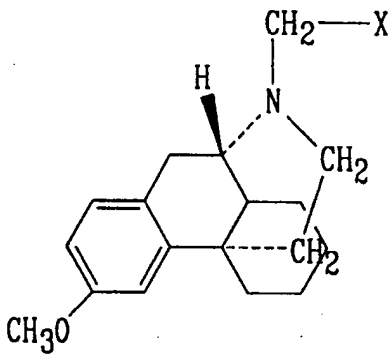


Chlorzoxazone derivative II

FIG 6



Dextromethorphan



Dextromethorphan derivative I

where X = $(CH_2)_nCOOH$

$(CH_2)_nNH_2$

$(CH_2)_nNHCOCH_2CH_2COOH$

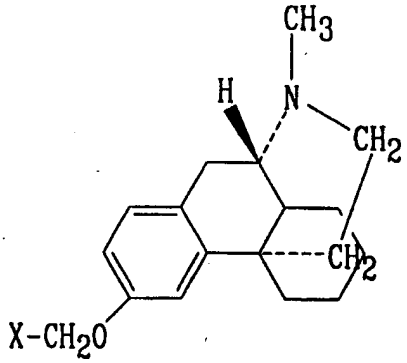
$(CH_2)_nN=C=S$

$(CH_2)_nOH$

$(CH_2)_nOCOCH_2CH_2COOH$

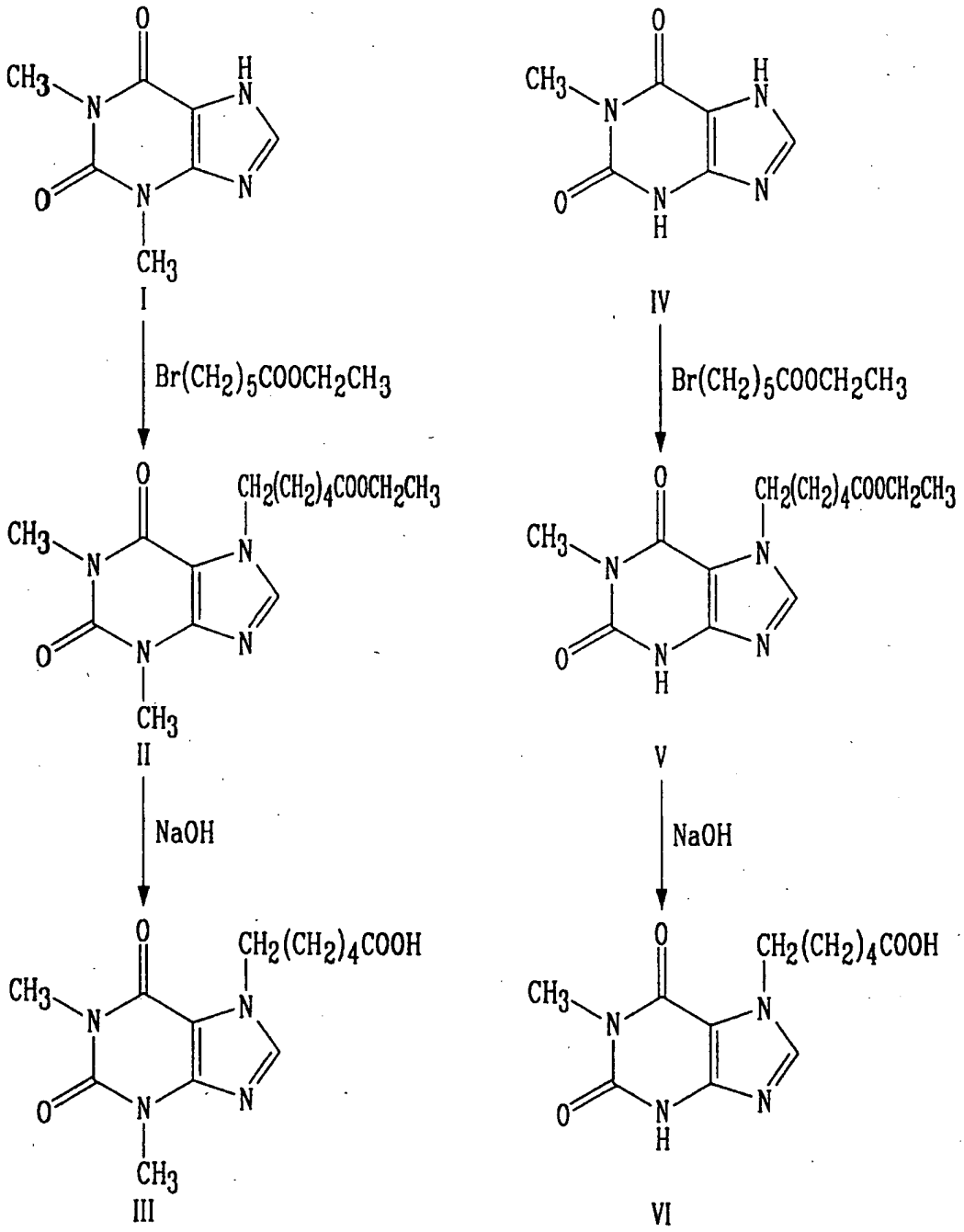
CH_2I

where n = 1-5



Dextromethorphan derivative II

FIG. 7



Caffeine derivative

1,7-dimethylxanthine derivative

FIG. 8

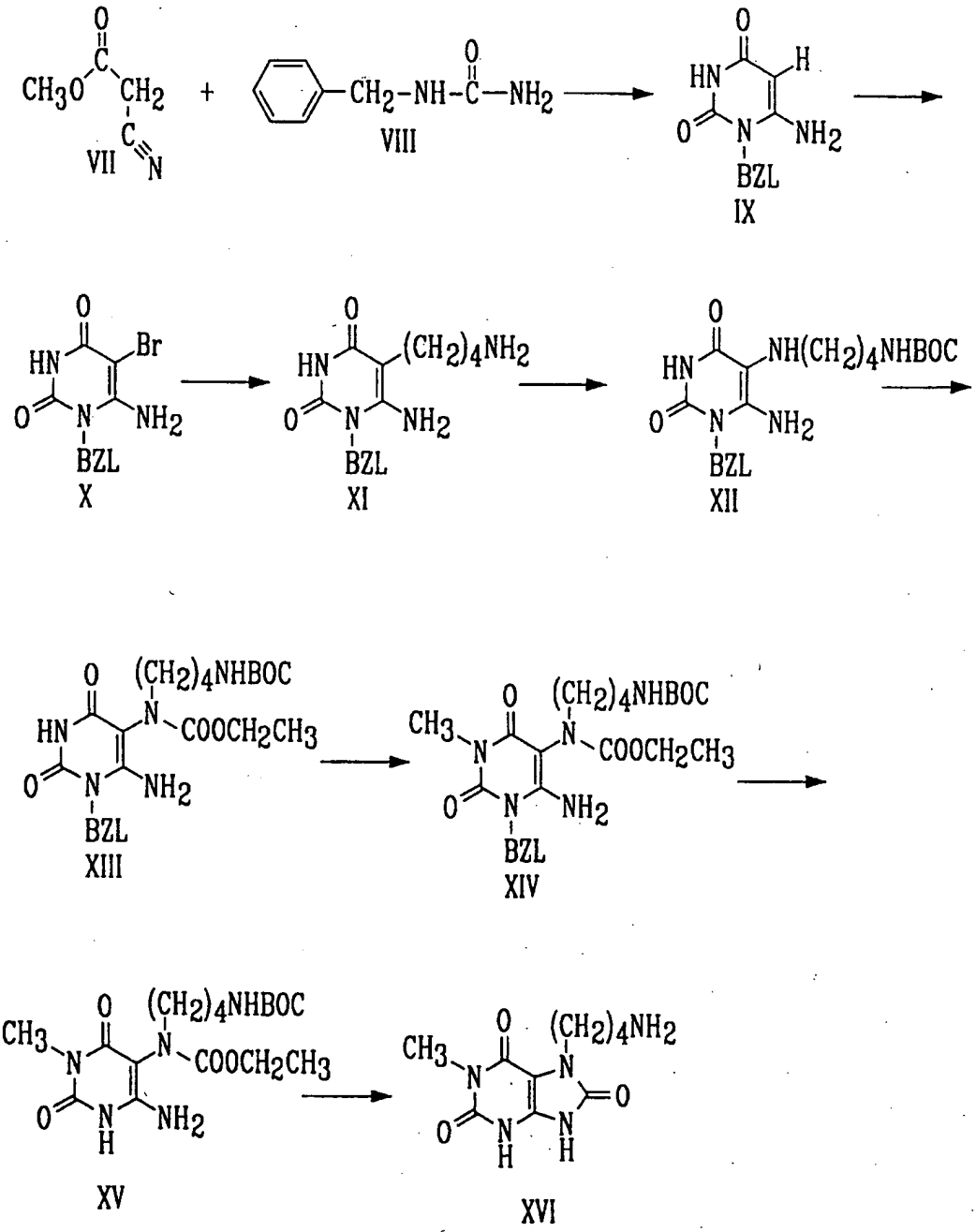

VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
1,7-dimethyluric acid derivative

Fig. 9

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	STD8	STD16	S1	S9	S5	S1	S9	S5	BIK	STD8	STD16
B	STD1	STD9	STD17	S2	S10	S6	S2	S10	S6	STD1	STD9	STD17
C	STD2	STD10	STD18	S3	S11	S7	S3	S11	S7	STD2	STD10	STD18
D	STD3	STD11	STD19	S4	S12	S8	S4	S12	S8	STD3	STD11	STD19
E	STD4	STD12	STD20	S5	S1	S9	S5	S1	S9	STD4	STD12	STD20
F	STD5	STD13	STD21	S6	S2	S10	S6	S2	S10	STD5	STD13	STD21
G	STD6	STD14	STD22	S7	S3	S11	S7	S3	S11	STD6	STD14	STD22
H	STD7	STD15	STD23	S8	S4	S12	S8	S4	S12	STD7	STD15	STD23

Fig - 10